ETHINYLESTRADIOL (EE2) DIFFERENTIALLY INTERFERES WITH INSULIN-LIKE GROWTH FACTOR I (IGF-I) IN LIVER AND EXTRAHEPATIC SITES OF MALE AND FEMALE BONY FISH

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Summary

During development growth and sexual differentiation are closely interrelated. In this context, the growth hormone (GH)/insulin-like growth factor (IGF)-I system and sex steroids are of critical importance. However, the potential interference of estrogen(s) and the GH/IGF-I system during development has not been investigated in fish. In order to study this potential interaction two different experimental approaches were chosen.

1. A population of tilapia was fed during 10-40 day post fertilization (DPF) with 17α-ethinylestradiol (EE2) enriched food. At 50, 75, 90 and 165 DPF, body length (BL), body weight (BW), sex ratio, serum IGF-I (radioimmunoassay) were measured. Pituitary GH mRNA, and IGF-I and estrogen receptor α (ERα) mRNA were quantitated in liver, brain, gonads and gills (real-time PCR) and the IGF-I mRNA results correlated to those of in situ hybridisation for IGF-I.

EE2 treatment led to a shift in sex ratio towards more females (165 DPF: 47.2 ± 8.5% females in control to 86.5 ± 14.1% in EE2 fed) and to severe progressive decrease in BL and BW in both sexes. Both parameters were significantly lowered from 90 DPF onwards (90 DPF - BL: -16.9% in males, -13.4% in females; BW: -47.4% in males, -32.3% in females; 165 DPF - BL: -19.5% in males, -15.2% in females; BW: -46.2% in males, -40.1% in females). Thus, developmental exposure to EE2 had persistent effects on sex ratio and growth.

At 75 DPF, in males the IGF-I serum level was significantly decreased in the EE2-treated group (5.65 ± 1.18 ng/mL) when compared to the controls (9.70 ± 2.06 ng/mL) while in females there was only a trend to reduce IGF-I serum level. In correlation, liver IGF-I mRNA was reduced at 75 DPF and recovered later. In situ hybridisation revealed a reduced number of hepatocytes containing IGF-I mRNA after EE2 treatment in male and female liver. In parallel to the suppression of IGF-I mRNA, ERα was transiently highly induced in both sexes, e.g. at 75 DPF, ERα mRNA was raised to the 2.2-fold in males and the 37-fold in females. Thus, growth impairment by estrogen(s) in fish may be due to prolonged direct suppression of liver IGF-I synthesis and release.

In female but not in male brain IGF-I mRNA was significantly suppressed at 75 DPF. In parallel, the number of neurones showing IGF-I mRNA was largely reduced in female

brain when compared to control. Brain ERα mRNA exhibited no significant changes at any experimental stage.

Pituitary GH mRNA was significantly decreased after EE2 treatment: in males at 165 DPF (2.33-fold) and in females at 75 DPF (2.27-fold) and at 90 DPF (3-fold). In parallel, ERα mRNA was significantly raised at 165 DPF (3.38-fold) in the male pituitary and in the female pituitary at 75 DPF (2.55-fold) and at 90 DPF (2.7-fold).

A transient downregulation of IGF-I mRNA occurred in ovaries (75 DPF) and testes (90 DPF). In agreement, in situ hybridisation revealed less IGF-I mRNA signals in granulosa and germ cells. In male gonad, a significant raise in ERα mRNA was found from 50 to 90 DPF but in female gonad ERα mRNA was significantly increased at 50 DPF followed by a significant decrease at 75 DPF.

In gills, IGF-I mRNA expression was significantly reduced only in males (50 DPF). In correspondence, the number of IGF-I mRNA containing chloride cells in the gill filament epithelium was reduced. ERα mRNA was significantly decreased in gills of both sexes only at 50 DPF.

In summary, the results of the EE2 feeding experiments show for the first time that developmental estrogen treatment impairs GH/IGF-I expression in fish, and that the effects persist. These long-lasting effects seem to be exerted both indirectly via inhibition of pituitary GH and directly by suppression of local IGF-I in organ-specific cells.

2. Because our experiments with feeding of high doses of EE2 during development indicated that estrogen treatment impairs GH/IGF-I expression in fish and that these effects persist, we, consecutively, studied the effects of exposure to low doses of EE2 to developing tilapia.

Female and male monosex populations were exposed to environmental concentrations (5 ng/L, 25 ng/L) of EE2 in the surrounding water (10-100 DPF). The actual EE2 concentrations after 36 h of exposure were somewhat lower than the applied concentrations (5 ng EE2/L aquaria: 4.34 ± 0.85 ng EE2/L; 25 ng EE2/L aquaria: 16.23 ± 5.74 ng EE2/L). The following parameters were determined at 30, 50, 75 and 100 DPF: sex ratio, BL, BW, and mRNA expression levels of IGF-I and ERα in liver, brain, gonads and gills (real-time PCR).

Sex ratio had shifted at 75 and 100 DPF after exposure to both 5 and 25 ng EE2/L. Both female and male monosex fish populations exhibited a higher percentage of females.

Growth was more pronouncedly reduced in male than in female fish. In males, from 50 DPF on both BL and BW were significantly decreased in fish exposed in either 5 or 25 ng EE2/L. At 100 DPF, BL was reduced by about 9.5% (5 ng EE2/L) and 11% (25 ng EE2/L)

and BW by about 25% (5 and 25 EE2/L). In females, both parameters showed only a trend to decrease.

In the liver of both sexes, IGF-I mRNA levels were significantly raised after exposure to both EE2 concentrations. This rise was accompanied by elevated ERα levels. At later stages (50–100 DPF), both IGF-I and ERα mRNA were decreased again. In general, the suppression of IGF-I expression was more pronounced in males than in females.

A sex difference was observed between the responses of male and female brain to EE2 exposure. In males, IGF-I mRNA was significantly suppressed only with 25 ng EE2/L (30, 50 DPF). ERα mRNA was significantly elevated at 30 DPF at both EE2 concentrations and decreased at 100 DPF. In females, IGF-I mRNA was suppressed (50, 75 DPF) under both EE2 concentrations and later recovered, and ERα mRNA was suppressed over the whole treatment phase with several significant stages.

A marked sex-specific difference was also found in the gonads. In testes, ERα was continuously, in part significantly, suppressed at 30 DPF (25 ng EE2/L) and at 50 and 100 DPF (5 ng EE2/L). The suppressed ERα mRNA expression levels were paralleled by continuously decreased IGF-I mRNA levels. In ovary, no significant changes in ERα mRNA were detected. The IGF-I mRNA levels showed a tendency to increase with time and were significantly upregulated at 100 DPF with both EE2 concentrations.

In gills, IGF-I mRNA expression was significantly declined only at 50 DPF (25 ng EE2/L) in both sexes. ERα mRNA was significantly increased in males at 30 and 75 DPF (25 ng EE2/L) while in females ERα mRNA was significantly decreased at 50 and 70 DPF.

In summary, even low concentrations of EE2 as can be found in the environment are capable of a significant impairment of growth and differentiation of developing fish. This again underlines the importance of the interactions between the estrogen and the GH/IGF-I system.

Zusammenfassung

Während der Entwicklung sind Wachstum und sexuelle Differenzierung eng miteinander verflochten. In diesem Zusammenhang sind Östrogen und das Wachstumshormon (GH)/Insulin-like growth factor (IGF)-I System von besonderer Bedeutung. Dennoch sind mögliche Interaktionen von Östrogen und dem GH/IGF-I System während der Entwicklung beim Fisch noch nicht untersucht worden. Um potentielle Interaktionen zu untersuchen, wurden zwei verschiedene experimentelle Ansätze gewählt.

1. Eine Tilapiapopulation wurde vom 10. bis 40. Tag nach Befruchtung (DPF) mit 17α-Ethinylestradiol (EE2)-angereicherter Nahrung gefüttert. An den Stadien 50, 75, 90 und 165 DPF wurden Körperlänge (KL), Körpergewicht (KG), Geschlechterverteilung und der IGF-I Serumspiegel (mittels Radioimmunoassay) gemessen. Die GH-mRNA Expression in der Hypophyse sowie IGF-I-und Östrogenrezeptor α (ERα) mRNA-Expression wurden in Leber, Gehirn, Gonaden und Kiemen (mittels Real-time PCR) bestimmt und die Ergebnisse für IGF-I mit den Resultaten einer IGF-I In situ-Hybridisierung verglichen.

Die Behandlung mit EE2 führte zu einer Verschiebung der Geschlechterverteilung zugunsten der Weibchen (165 DPF: 47.2 ± 8.5% Weibchen in der Kontrollgruppe gegenüber 86.5 ± 14.1% bei den mit EE2 gefütterten Tieren) und zu schweren progressiven Beeinträchtigungen von KL und KG bei beiden Geschlechtern. Beide Parameter waren signifikant erniedrigt beginnend mit dem Stadium 90 DPF (90 DPF - KL: -16.9% bei Männchen, -13.4% bei Weibchen; KG: -47.4% bei Männchen, -32.3% bei Weibchen; 165 DPF - KL: -19.5% bei Männchen, -15.2% bei Weibchen; KG: -46.2% bei Männchen, -40.1% bei Weibchen). Somit hatte die Exposition gegenüber EE2 während der Entwicklungsphase dauerhafte Veränderungen der Geschlechterverteilung und des Wachstums zur Folge.

Am 75 DPF war bei den Männchen der IGF-I Serumspiegel signifikant vermindert in der EE2-behandelten Gruppe (5.65 ± 1.18 ng/L) im Vergleich zu den Kontrolltieren (9.7 ± 2.06 ng/L) während bei den Weibchen lediglich ein Trend zu einem erniedrigten IGF-I Serumspiegel bestand. Dementsprechend war die IGF-I mRNA Expression in der Leber am 75 DPF reduziert und erholte sich später. Die In situ-Hybridisierung zeigte in der männlichen und weiblichen Leber nach EE2-Behandlung eine verminderte Anzahl von Hepatozyten, die IGF-I mRNA enthielten. Parallel zur Erniedrigung der IGF-I Genexpression war die ERα Genexpression vorübergehend stark erhöht, beispielsweise am 75 DPF auf das 2.2fache bei

Männchen und das 37fache bei Weibchen. Somit könnte die Beeinträchtigung des Wachstums durch Östrogene beim Fisch auf einen verlängerten direkten supprimierenden Effekt auf die IGF-I Synthese und Freisetzung durch die Leber zurückzuführen sein.

Im weiblichen, nicht aber im männlichen Gehirn, war die IGF-I Genexpression bei Stadium 75 DPF signifikant supprimiert. Dementsprechend war zu diesem Zeitpunkt die Anzahl von Neuronen, die IGF-I mRNA aufwiesen, beim weiblichen Gehirn deutlich reduziert im Vergleich zu den Kontrolltieren. Bei keinem Entwicklungsstadium zeigte die ERα Genexpression im Gehirn signifikante Veränderungen.

Bei beiden Geschlechtern war die GH mRNA Expression in der Hypophyse nach EE2-Behandlung signifikant erniedrigt bei den Männchen am 165 DPF (2.33fach) und bei den Weibchen am 75 DPF (2.27fach) und 90 DPF (3fach). Gleichzeitig war die ERα Genexpression signifikant erhöht, am 165 DPF (3.38fach) in der männlichen Hypophyse und am 75 DPF (2.55fach) und 90 DPF (2.7fach) in der weiblichen Hypophyse.

Eine vorübergehende Erniedrigung der IGF-I mRNA Expression erfolgte in den Ovarien (75 DPF) und Hoden (90 DPF). In Übereinstimmung damit zeigte auch die In situ-Hybridisierung weniger IGF-I mRNA Signale in den Granulosa- und Keimzellen. In der männlichen Gonade wurde ein signifikanter Anstieg der ERα mRNA in den Stadien 50-90 DPF gefunden, während in der weiblichen Gonade die ERα mRNA signifikant erhöht war am 50 DPF, aber gefolgt von einem signifikanten Abfall am 75 DPF.

In den Kiemen wurden signifikante Veränderungen der IGF-I mRNA Expression nur bei den Männchen (50 DPF) erzielt. Dementsprechend war die Anzahl der IGF-I mRNA-enthaltenden Chloridzellen im Kiemenepithel vermindert. Nur am 50 DPF war bei beiden Geschlechtern die ERα mRNA signifikant erniedrigt.

Zusammenfassend kann festgehalten werden, dass die Ergebnisse der Experimente mit Fütterung von EE2 zum ersten Mal zeigen, dass die Behandlung mit Östrogen während der Entwicklung die GH/IGF-I Expression beim Fisch beeinträchtigt, und dass diese Effekte fortbestehen. Die nachhaltigen Effekte scheinen sowohl indirekt über die Erniedrigung von Wachstumshormon in der Hypophyse als auch direkt über die Unterdrückung des lokalen IGF-I in den organ-spezifischen Zellen zu wirken.

2. Da unsere Fütterungs-Experimente mit hochdosiertem EE2 darauf hindeuteten, dass Östrogen-Behandlung während der Entwicklung die GH/IGF-I Expression beim Fisch beeinträchtigt, und dass diese Wirkungen fortbestehen, haben wir daraufhin die

Auswirkungen einer Exposition gegenüber niedrigen EE2-Dosen auf Tilapien während der Entwicklung untersucht.

Weibliche und männliche Monosex-Populationen von Tilapien wurden Umweltkonzentrationen (5 ng/L, 25 ng/L) von EE2 während 10-100 DPF ausgesetzt. Messungen der aktuellen EE2-Konzentrationen nach 36 Stunden Exposition ergaben, dass diese niedriger als die applizierten Dosen waren (5 ng EE2/L Aquarien: 4.34 ± 0.85 ng EE2/L, 25 ng EE2/L Aquarien: 16.23 ± 5.74 ng EE2/L). Folgende Parameter wurden zu den Stadien 30, 50, 75 und 100 DPF bestimmt: Geschlechterverteilung, KL, KG, und die Genexpressionsveränderungen von IGF-I und ERα mRNA in Leber, Gehirn, Gonaden und Kiemen (Real-time PCR).

Die Geschlechterverteilung war verschoben (75 und 100 DPF). Sowohl die weibliche als auch die männliche Monosex-Population zeigten einen höheren Anteil an Weibchen nach Exposition gegenüber 5 und 25 ng EE2/L. Das Wachstum war bei den Männchen stärker reduziert als bei den Weibchen. Bei den Männchen waren KL und KG vom Stadium 50 DPF an bei den Fischen, die gegenüber 5 und 25 ng EE2/L exponiert waren, signifikant reduziert. Am Ende des Experimentes (100 DPF) war die KL um ca. 9.5% (5 ng EE2/L) bzw. 11% (25 ng EE2/L) vermindert und das KG um ca. 25% (5 und 25 ng EE2/L). Bei den Weibchen zeigten beide Parameter lediglich eine tendenzielle Reduktion.

Bei beiden Geschlechtern war die Expression von IGF-I mRNA in der Leber nach Exposition gegenüber beiden EE2-Konzentrationen signifikant erhöht. Dieser Anstieg war von einer erhöhten ERα Genexpression begleitet. In den späteren Untersuchungsstadien (50-100 DPF) waren sowohl IGF-I- als auch ERα Genexpression wieder erniedrigt. Im Allgemeinen war die Unterdrückung der IGF-I Genexpression bei den Männchen stärker ausgeprägt als bei den Weibchen.

Die Reaktionen des männlichen und weiblichen Gehirns gegenüber der EE2-Behandlung zeigten deutliche Unterschiede. Bei den Männchen war die IGF-I mRNA Expression nur bei 25 ng EE2/L (30 und 50 DPF) signifikant erniedrigt. Die ERα mRNA war signifikant erhöht bei EE2 Exposition (30 DPF) und später erniedrigt (100 DPF). Bei den Weibchen war die IGF-I Genexpression bei beiden EE2-Konzentrationen supprimiert (50 und 75 DPF) und kehrte später zu den Ausgangswerten zurück. Bei beiden EE2-Konzentrationen zeigte die ERα Genexpression eine – mitunter signifikante – Absenkung während der gesamten Behandlungsdauer.

Eine bemerkenswerte geschlechtsspezifische Reaktion wurde auch in den Gonaden festgestellt. Im Hoden war die ERα Genexpression kontinuierlich, teilweise signifikant,

erniedrigt, nämlich an den Stadien 30 DPF (25 ng EE2/L) sowie 50 und 100 DPF (5 ng EE2/L). Die supprimierten ERα Genexpressionswerte waren dauerhaft begleitet von einer erniedrigten IGF-I Genexpression. Im Ovar wurde keine signifikante Veränderung der ERα Genexpression gemessen. Die IGF-I Genexpressionswerte zeigten einen tendenziellen Anstieg mit Fortdauer der Exposition und waren bei beiden EE2-Konzentrationen am 100 DPF signifikant erhöht.

In den Kiemen wurde ein signifikanter Abfall der IGF-I mRNA Expression nur am 50 DPF (25 ng/L) bei beiden Geschlechtern gefunden. Die ERα Genexpression war bei den Männchen an den Stadien 30 und 75 DPF (25 ng/L) signifikant erhöht, während sie bei den Weibchen an den Stadien 50 und 75 DPF signifikant erniedrigt war.

Zusammenfassend kann festgestellt werden, dass sogar niedrige Konzentrationen von EE2, wie sie in der Umwelt gemessen werden, in der Lage sind, Wachstum und Differenzierung von Fischen während der Entwicklung signifikant zu beinträchtigen. Dies unterstreicht die Bedeutung der Interaktionen des Östrogen- und des GH/IGF-I-Systems.

1. Introduction

1.1. The endocrine disruption hypothesis

The endocrine disruption hypothesis claims that synthetic as well as naturally occurring chemical substances in the environment can disrupt the normal functions of the endocrine system and its hormones in humans and wildlife. Krimsky S., in his book "Hormonal Chaos" (2000), claims that "from the standpoint of human pathology, the environmental endocrine hypothesis could turn out to be the most significant environmental health hypothesis since the discovery of chemical mutagenesis." During the last 20 years this hypothesis has received increasing scientific and public attention (Colborn and Clement, 1992; McLachlan, 2001; Matthiessen, 2003). A number of adverse alterations observed in wildlife, e.g. the disturbed sexual development in birds (Fry and Toone, 1981) or the occurrence of intersex in wild fish (Jobling et al., 1998), have been thought to be caused by exposure to endocrine-disrupting chemicals (EDCs). Over the past decade scientists have reported high levels of deformities and defects in fish eggs and larvae from the North sea (Dethlefsen et al., 1996), increased incidents of idiopathic hepatic lesions in English sole (*Parophrys vetulus*) from urban areas in the United States (Myers et al., 1991) and "feminisation" of male fish near sewage treatment outlets in rivers and estuaries (Sumpter and Jobling, 1995).

The seriousness of the problem has led the Organisation for Economic Cooperation and Development (OECD) and the European Union to initiate extended research programs towards new guidelines and regulations on EDCs. An EDC is defined by the World Health Organisation (2002) as "an exogenous substance or mixture that alter function(s) of the endocrine system and consequently produces adverse health effects in an intact organism, or its progeny, or (sub)populations".

1.2. Estrogenic acting compounds found in the environment

Organisms have evolved sensitivity to endogenous and exogenous chemicals in order to respond to physical or biological stimuli in the environment and to maintain internal homeostasis, however, this sensitivity at the same time makes organisms vulnerable to

inadvertent signals in their environment (Cheek et al., 1998). Through their effects on metabolism, distribution or function of endogenous hormones, EDCs have the potential to disrupt hormone-controlled physiological processes such as development, growth, stress responses, sexual differentiation or reproduction. A variety of environmental substances has been shown to interfere with the endocrine system of exposed organisms either by mimicking hormone action or by disturbing hormone synthesis and metabolism (Segner et al., 2006).

Examples of environmental EDCs include natural estrogens (e.g. from human excretion), synthetic estrogens (e.g. from contraceptives), phytoestrogens (e.g. from soy or wood-processing), mycoestrogens, chlorinated pesticides, such as dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), polybrominated flame retardants and other industrial chemicals such as alkylphenols or phtalates, plastics, detergents, drugs, cosmetics, herbicides, and others, entering the environment through industrial and sewage discharges, active application and runoff. A lot of "inadvertent" estrogens are much less potent than the steroidal estrogen, 17β-estradiol (E2) (McLachlan, 2001). Like all steroid hormones, E2 contains the three-ring phenanthrene; whereby for estrogenicity, the first or A ring must contain a phenolic hydroxyl group (Fig. 1).

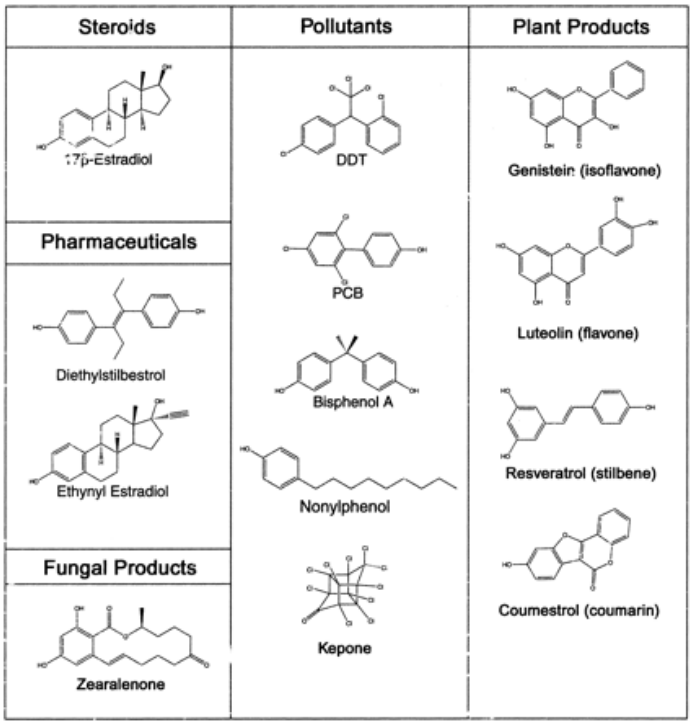

Fig. 1 Chemicals found in the environment reported to be estrogenic (McLachlan, 2001).

In 1938, diethylstilbestrol (DES) was discovered by Sir Charles Dodds and colleagues (Dodds et al., 1938). It was orally active, relatively stable, and less expensive to produce than

isolated or synthesized steroidal estrogens. DES quickly became the synthetic estrogen of choice for medicine and, later, agriculture. Its medical uses included estrogen replacement therapies, lactation suppression, postcoital contraception, pregnancy maintenance, and prostate cancer therapy. In agriculture, DES was mainly used to chemically caponize chicken and stimulate growth in cattle. The widespread use of DES in cattle feed lots led to the introduction of tons of potent estrogens into the ecosystem. Its use was discontinued in 1971 after a report that associated *in utero* DES exposure with vaginal clear cell adenocarcinoma, in a small number (~0.1%) of daughters of women who had taken the drug during pregnancy (Herbst et al., 1971). Subsequent studies in women exposed to DES have detected nonneoplastic abnormalities such as anatomical malformations of the cervix, vagina, and uterus as well as decreased fertility and less successful pregnancies (Mittendorf, 1995; Herbst, 2000). Experiments with male and female ERα knockout mice have indicated that DES elicits its effects through an ERα-dependent signalling pathway (Henley and Korach, 2006).

Very recently, a synthetic estrogenic component of commonly used oral contraceptives, 17α-ethinylestradiol (EE2), was found in waste water effluent in Europe (Kalbfus, 1995; Harries et al., 1997; Desbrow et al., 1998; Larsson et al., 1999; Baronti et al., 2000; Lagana et al., 2000). EE2 is even more potent than the naturally occurring E2. Purdom et al. (Purdom et al., 1994) have reported that EE2 at a concentration of as low as 0.1 ng/L affects the reproductive function of male rainbow trout and at a concentration of 10 ng/L induced the synthesis of vitellogenin (VTG) in immature cyprinids. Lange and colleagues (Lange et al., 2001) have demonstrated that the no-observed-effect concentrations (NOEC) for growth, survival, and reproduction (as eggs production) for the F_0 adult phase and F_1 larval survival were ≥1.0 ng EE2/L. In a recent study in Canada authors were unable to detect any EE2 in the wastewater treatment plants (WWTP) samples by YES bioassay and E-Screen bioassay (Nelson et al., 2007). However, concentrations of EE2 effluents in Germany had been found in the range of 0.3-0.5 ng/L (Kalbfus, 1995). In the UK, EE2 concentrations in effluents were up to 7 ng/L (Desbrow et al., 1998), while Larsson et al. (Larsson et al., 1999) reported Swedish sewage treatment works effluent to contain EE2 at up to 4.5 ng/L in contrast to the traces amounts reported by Lagana et. al. (2000) for Italy. Baronti et al. (2001) have shown that mean EE2 concentrations in Roman WWTP influent and effluent were 3 ng/L and 0.4 ng/L, respectively. Layton et al. (2000) also have reported that as much as 80% of the EE2 may be bound to the sewage sludge and thus be removed from the aqueous phase.

The most commonly studied phytochemicals are the flavonoids, including isoflavones and flavones, represented by Genistein and Luteolin, respectively. It has been shown that

isoflavones, like Genistein, bind to vertebrate forms of ERα or ERβ and alter the transcription of estrogen-responsive genes (Kuiper et al., 1998). Genistein also has potent tyrosine kinase-inhibiting effects (Zava and Duwe, 1997). Also Zearalenone, a fungal mycotoxin produced by *Fusarium*, binds to the ER (Powell-Jones et al., 1981), was uterotropic in the newborn rat (Sheehan et al., 1984), impaired fertility in cattle (Roine et al., 1971), and induced hyperestrogenicity in swine (Bristol and Djurickovic, 1971). Studies on sheep exposed to the phyto-estrogen Coumestrol displayed macroscopic changes within the genital tract, of which the uterine alterations were especially prominent (Cantero et al., 1996). Resveratrol is a phytoestrogen that has the ability to bind to ERs and evoke biological effects like estrous cycle irregularity, ovarian hypertrophy, and alterations in sociosexual behavior (Henry and Witt, 2006).

The best known example for "inadvertent" chemicals is the pesticide Dichlorodiphenyltrichloroethane (DDT). In 1950 Burlington and Lindeman (Burlington and Lindeman, 1950) showed that DDT can estrogenize cockerels. Later Conney and colleagues (Conney et al., 1967) rediscovered it while testing various pesticides as inducers of cytochrome P450 enzyme activity.

In addition to DDT, other chlorinated hydrocarbons, PCBs, have been shown to function estrogenic both by *in vivo* and *in vitro* assays (Korach et al., 1988).

Nonylphenol (NP) is an industrial chemical found in a variety of commercial products. It belongs to a group of synthetic compounds that mimic the effect of estrogens by binding to the ER (Flouriot et al., 1995). NP induces VTG synthesis in various fish species (Sumpter and Jobling, 1993; Madsen et al., 1997; Christiansen et al., 1998). Soto et al. (1991) showed that some plastic petri dishes and tubes contained a residue of p-nonylphenol. Thus, this alkylphenol contaminant has been shown to be estrogenic, supporting the association between p-nonylphenol contamination and feminisation of fish in UK streams (Sumpter and Jobling, 1993).

Bisphenol-A (BPA) was found to be an efficient cross-linking chemical and came to be widely used in the production of plastic polymers, primarily polycarbonates. In 1993, the BPA production in the USA amounted to 640,000,000 kg; of that, 44,000 kg (<0.10%) were recycled, land filled, incinerated, or released into the environment (McLachlan et al., 2001). Levels of phenolic xenoestrogens in river water can be surprisingly high. Measured concentrations of BPA, 4-*tert*-octylphenol and the technical isomer mixture of 4-nonylphenol in the river water samples from Germany ranged from 20 to 1927 ng/L, <10 to 770 ng/L and <10 to 420 ng/l, respectively (Quednow et al., 2007). Similar analyses from Turkish rivers

found that alkylphenols were not present in the water but concentrated in the sediments (1–4.46 μg/g) (Uguz et al., 2003). Higher values have been reported from England (15 μg/g) and the USA (70 μg/g) (Blackburn et al., 1999). As would be expected for these lipophilic compounds, analysis revealed that alkylphenols contaminate the tissues of fish. Values of 0.1–0.8 μg/g NP have been estimated for fish sampled from both English and Turkish rivers (Blackburn et al., 1999; Uguz et al., 2003).

At least in summer months, fish may also be contaminated with camphor derivatives from sun-screen creams used by people swimming in lakes and rivers (Poiger et al., 2004). The levels in fat tissue in fish from the Meerfelder Maar (Germany) were similar to the concentration of 7 mg/kg, which has been reported to affect development and reproduction in medaka (Inui et al., 2003).

Phthalate esters are used extensively as plasticizers and stabilizers in a variety of plastics and consumer goods. Exposure to phthalates through ingestion, inhalation, and dermal absorption occurs throughout life (Latini, 2005). Phthalate esters, including di(*n*-butyl) phthalate (DBP) (Fig. 2), adversely affect the male rat reproductive tract both after prenatal or postnatal exposure. The effects include disrupted epididymal development, hypospadia, cryptorchism, and reduced fertility. Furthermore, neither DBP nor its major metabolite, monobutyl phthalate, physically interact with the androgen receptor (AR), indicating that the antiandrogenic effects of DBP occur through AR-independent mechanisms (Foster et al., 2001).

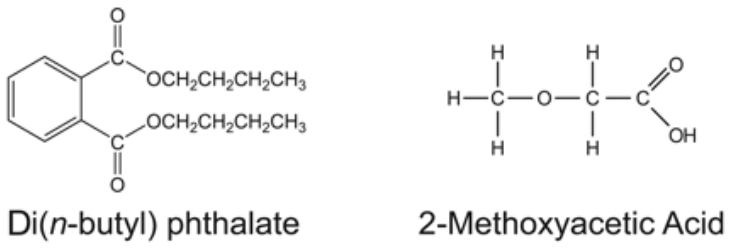

Fig. 2 The chemical structures of selected EDCs.

In general, in EDCs, there is often, but not always, the presence of an aromatic ring or two. Several representative chemicals contain chlorine atoms. The role that chlorine plays in hormonal activity is still not clear. For instance Kepone (Fig. 1), a structurally restricted, cubic molecule containing chlorine on every carbon but one, is known to be estrogenic (Eroschenko and Palmitter, 1980).

Methoxyacetic acid (MAA) (Fig. 2) is the major metabolite of ethylene glycol monomethyl ether (EGME), an industrial solvent commonly used in varnishes, paints, dyes, and fuel additives (Johanson, 2000). Occupational exposure to both EGME and MAA has been associated with subfertility, spontaneous abortion, and reduced sperm counts (Welch et al., 1988; Ratcliffe et al., 1989; Correa et al., 1996). MAA exerts its effects by potentiating the ligand-induced transcriptional activity of ERα, ERβ, progesterone receptor (PR), AR, and the thyroid hormone receptor (TRβ). By this potentiation even weak nuclear receptor agonists found in the environment are able to produce responses of full agonists (Henley and Korach, 2006).

As yet, no synthetic environmental chemicals have been reported that function as androgens, but twenty years ago, Howell and colleagues (1980) described masculinization of female mosquito fish*, Gambusia affinis holbrooki,* which were caught downstream from the effluent discharged from a paper mill in Cantonment, Florida. *Mycobacterium smegmatis*, which produces androgens as metabolic products, had formed extensive colonies in the effluent path and was utilizing the plant cholesterol (stigmasterol) as carbon sources and metabolizing the plant sterol to a potent androgen, androstenedione.

A growing number of pesticides have been recently recognized as androgen antagonists. The organophosphate insecticide, Fenitrothion, has also been shown to be a competitive reversible inhibitor of the AR (Tamura et al., 2001). The herbicide Linuron has turned out to be a competitive binding ligand for the AR in rats or humans, and altered androgen-dependent gene expression in castrate rats (Lambright et al., 2000). Gray et al. (1999) demonstrated that Vinclozolin produces subtle alterations in the differentiation of the external genitalia, ventral prostate, and nipple tissue in male rat offspring. Vinclozolin is metabolized to at least two active forms, that display antiandrogenic activity by binding to the AR.

1.3. Genomic and non-genomic mechanisms of ED action

The biological actions of hormones, such as estrogens, progesterone, androgens, and thyroxine, are mediated via high affinity protein receptors within the target cells. Steroids, such as the estrogens, normally circulate as their sulphated derivatives which have no hormonal effects; the free steroid is released at the target tissue by the action of tissue-bound sulphatase enzymes. Other steroids circulate in the blood stream bound to carrier proteins or

serum albumin. All steroids are fat-soluble and readily cross the cell membrane, interacting with dimeric receptor proteins; in the case of estrogens these are ER-α and ER-β, although recent evidence has been provided for a third class, ER-γ, in fish and, possibly, mammals (Dodge et al., 1996; McLachlan et al., 2001). The affinity of a steroid for its receptor is high. The steroid-receptor complex binds to target regions of DNA termed "response elements". This activates a cascade of reactions. Classically, EDCs have been thought to exert their effects exclusively by genomic mechanisms, acting as steroid agonists or antagonists by binding to the receptor. However, there is evidence that some estrogenic compounds do not act via the ER and thus non-genomic effects may also play a role in the mechanisms of action of EDCs (Fig. 3).

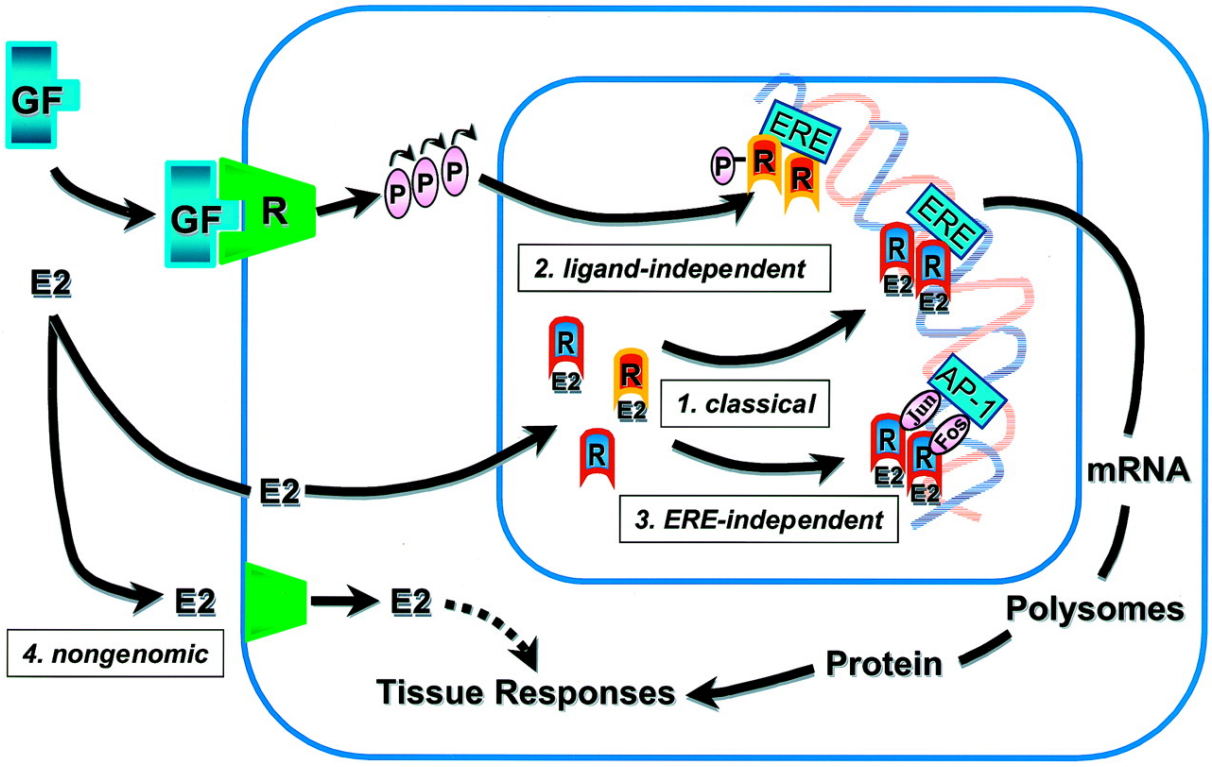

Fig. 3 The multiple mechanisms of estradiol (E2) and estrogen receptor (ER) signalling. The biological effects of E2 are mediated through at least four ER pathways. *1*, *classical ligand-dependent*, E2-ER complexes bind to EREs in target promoters leading to an up- or downregulation of gene transcription and subsequent tissue responses. *2, ligand-independent*. Growth factors (*GF*) or cyclic adenosine monophosphate (not shown) activate intracellular kinase pathways, leading to phosphorylation (*P*) and activation of ER at ERE-containing promoters in a ligand-independent manner. *3*, *ERE-independent*, E2-ER complexes alter transcription of genes containing alternative response elements such as activator protein 1 (AP-1) through association with other DNA-bound transcription factors (Fos/Jun), which tether the activated ER to DNA, resulting in an up-regulation of gene expression. *4*, *Cell-surface (nongenomic) signaling*, E2 activates a putative membraneassociated binding site, possibly a form of ER linked to intracellular signal transduction pathways that generate rapid tissue responses. (Hall et al., 2001).

Classical genomic effects (ligand-dependent, ligand-independent and ERE-independent) have been demonstrated for natural compounds (e.g. coumestrol),

pharmaceuticals (e.g. tamoxifen and DES) and industrial chemicals (e.g. octylphenol and BPA). These compounds bind to the ER and thus act as pseudoestrogens, resulting in feminising effects (Laws et al., 1995; Gray and Metcalfe, 1999; Stroheker et al., 2004).

Goksøyr A. (2006) divided the mechanisms of action of EDCs into: (1) agonist/antagonistic effect ("hormone mimics"), (2) disruption of production, transport, metabolism or secretion of natural hormones, and (3) disruption of production and/or function of hormone receptors (Goksøyr et al., 2003). Results from his laboratory indicate that one single compound can utilize all of these mechanisms, apparently depending on the doses given to the organism. Studies with Atlantic salmon (*Salmo salar*), have shown that 4-nonylphenol can act as estrogen mimic (Arukwe et al., 1997a), steroid metabolism disruptor (Arukwe et al., 1997b), and by modulating ER levels (Yadetie et al., 1999).

EDCs may also exert non-genomic effects by altering synthesis or availability of endogenous hormones. Steroids are initially formed from cholesterol via a series of reactions, which can involve cytochrome P450 (CYP) isoforms. CYP 2C11, 2A1, 2B1, 3A1 and 2C19 catalyse critical stages; CYP 2C19 is the enzyme 'aromatase' which controls the formation of estrogens from precursors. Expression of CYPs from the 2B and 2C families occurs during the earliest stages of fetal development (You, 2004). Since they can be induced by environmental contaminants such as DDT and its analogues, this gives another mechanism by which EDC activity can occur. Compounds of the azole type, such as ketoconazole and the fungicide fenarimol, inhibit these CYP isoforms and consequently can also affect steroid synthesis (Hirsch et al., 1987). The now-banned anti-fouling agent tributyltin and its metabolites, are also thought to act by inhibition of aromatase (Alzieu, 2000).

Estrogens and their steroid precursors are normally transported in the bloodstream as sulphonate esters. The esters are synthesised, using the cofactor 3′-phosphoadenosine-5′-phosphosulphate, by cytosolic sulphotransferase enzymes with the isoforms SULT 1E1 and SULT 2A1. Sulphatases located on the surfaces of the target cells release the free steroids, which then can enter the cell. Any excess steroids are reconjugated and excreted from the cells. The importance of this pathway has been shown by inserting the gene for SULT 1E1 into the MCF-7 breast cancer cell line resulting in a decrease of the proliferative response of the cells towards E2 (Falany et al., 2002). Hence, compounds which alter the sulphotransferase/sulphatase activity ratio can potentially affect the availability of endogenous estrogens to target tissues (Kirk et al., 2001). Many phenolic compounds, such as the alkylphenol plasticisers, inhibit both SULT 1E1 and SULT 2A1, and also act as substrates for SULT 1A1. Although this isoform mainly sulphonates phenols, it accepts estrogens as

substrates at relatively high concentrations and sulphonates alkylphenols at sub-micromolar levels (Harris et al., 2005). However, alkylphenols can cause significant inhibition of sulphotransferase activity at sub-micromolar concentrations, but some naturally occurring compounds, e.g. some types of flavonoids, are pronouncedly more potent (Kirk et al., 2001).

1.4. Estrogen receptors in fish

ERs are ligand-dependent transcription factors that regulate the expression of their target genes either directly, by binding to a specific *cis*-element called the estrogen-responsive element (ERE) in the promoters of target genes, or indirectly, via interactions with other transcription factors such as Sp1 or the AP1 complex (Hall et al., 2001). The ERs are divided into six functional domains designated A to F (Krust et al., 1986). The C domain (DNA binding-domain; DBD), and the E domain (ligand binding-domain; LBD), responsible for ligand binding, nuclear localization, and transcriptional activation (AF-2), are highly conserved with homologies of 90% and 100% respectively, between chicken, rat and man (Pakdel et al., 1989). In contrast, considerable divergence occurs in the N-terminal A/B domain, which contains a second activation function, AF-1, the D domain (hinge region), necessary for the maintenance of the three-dimensional structure of the ER (Zilliacus et al., 1995; Hu and Lazar, 1999), and the C-terminal F domain.

Recently, three estrogen receptors, ERα, ERβ and ERγ, have been identified in teleosts (Todo et al., 1996; Tchoudakova et al., 1999; Hawkins et al., 2000; Socorro et al., 2000, Kim et al., 2002). However, the biological significance of the multiple ERs still has to be fully elucidated. ERγ is closely related to ERβ, suggesting that it reflects a duplification of this gene. In zebrafish (*Danio rerio*), the ERα subtype is now designated ER1, the ERβ1 subtype ER2b, and the ERβ2 subtype ER2a (Official Zebrafish Nomenclature Guidelines; http://zfin.org). Distinct patterns of expression for ER1 and ER2 have been documented in fish, but these patterns appear to vary between species. In Atlantic croaker (*Micropogonias undulates*), goldfish (*Carassius auratus*), seabream (*Sparus aurata*) and zebrafish, ER1 and ER2 exhibit broad tissue expression but, for the most part, are concentrated in liver and gonad, with considerably lower levels of expression in brain, pituitary, intestine, and muscle (Tchoudakova et al., 1999; Hawkins et al., 2000; Socorro et al., 2000; Menuet et al., 2002). In contrast to these findings, however, ER1 in African cat-fish (*Clarias gariepinus*) and ER2a in goldfish have been reported to be expressed predominantly in pituitary and brain, with

considerably lower levels of expression in gonads and liver (Ma et al., 2000; Choi and Habibi, 2003; Teves et al., 2003). In fathead minnow (*Pimephales promelas*), ERs have been found to be expressed in brain, pituitary, liver, gonads, intestine, and gill of male and female fish, and ER2b and ER2a also in muscle. ER1 and ER2b were expressed predominantly in the liver, whereas ER2a was mainly expressed in intestine and lowest in liver. This differential expression is supported by the recent finding that fathead minnow exposed to 100 ng E2/L showed a significant 5-fold induction of ER1 in male liver but no effect was found on ER2b or ER2a expression (Filby and Tyler, 2005).

1.5. Effects of EDCs

1.5.1. In mammals

Studies in mammals have shown that reproductive function can be affected by compounds of both natural and industrial origin. Phytoestrogens from clover have long been recognised as the cause of reproductive dysfunction and infertility in sheep and the compounds responsible have been identified as equol and coumestrol (Rossiter and Beck, 1966). Industrial chemicals can also act as EDCs in mammals. Phthalate esters such as diethylhexylphthalate (DEHP) are major components in polyvinyl chloride (PVC) plastics, which are frequently used in medical tubing and blood storage bags. DEHP is readily released from medical devices, and DEHP and its metabolites have been detected in urine from babies after intravenous infusions in a neonatal intensive care unit (Calafat et al., 2004). DEHP is an endocrine disrupter in rats, producing Leydig cell hyperplasia and also affecting systemic physiology (Akingbemi et al., 2004). The main metabolite of DEHP, monoethylhexylphthalate (MEHP) acts in rats as an anti-estrogen at low doses but has estrogenic effects at higher levels (Shono and Suita, 2003). Di-*n*-butylphthalate (DBP) similarly reduced fertility in rabbits, whereby the effects became particularly apparent in the intrauterine period (Higuchi et al., 2003). Other plasticisers, such as nonyl- and octylphenols and their derivatives, also bind to the ERs. BPA also acts at the ERs in reproductive tissues and in high doses causes reproductive toxicity in rats and mice (Tan et al., 2003). Some UV filters in sunscreen creams are also estrogenic: 4-methylbenzylidene camphor and 3-methylbenzylidene camphor are ligands for both ER subtypes, particularly ERα, and have shown to cause estrogenic effects in rats (Schlumpf et al., 2004; Durrer et al., 2005).

Also for human, exposure to EDCs has been discussed as possible cause of changes in sperm quality and quantity (Sharpe and Skakkebaek, 1993) or as cause of increases in the number of some types of breast, prostate and testicular cancer (Colborn et al., 1993; Safe and Zacharewski, 1997). Also increases in the prevalence of cryptorchism and hypospadia have been reported (Dolk et al., 2004).

1.5.2. In birds, reptiles and amphibians

Unlike mammals, sexual differentiation in birds depends on estrogen levels which regulate the development of the female phenotype. Feminised male birds with ovotestis and reduced male copulatory behaviour have been induced by EDCs such as DDT which are agonists at the ER (Fry and Toone, 1981).In the 1960s the use of DDT coincided with a decrease in reproductive ability of birds, particularly those at the top of the food chain. Egg-laying and calcification of the egg shell in birds depends on estrogen and Vitamin D. For example, the presence of organochlorines not only resulted in feminisation of male Japanese quail but also led to shells which were too fragile to hatch successfully or so thick that the chicks could not get out (Halldin et al., 2003).

EDC effects have also been documented in alligators in Florida, where exposure to DDT and its metabolite DDE markedly decreased the egg hatching rate and increased juvenile mortality (Vonier et al., 1996). The surviving animals showed raised estradiol levels in both sexes. DDT and other organochlorines are now banned over most of the world.

The commercially important weed killer atrazine has been known to exert estrogenic effects on tadpoles and adult frogs, to convert adult males into hermaphrodites and to retard gonadal development (Hayes et al., 2003). Furthermore, the exposure to exogenous steroids or steroid mimicking chemicals led to feminisation or masculinisation of *Xenopus laevis* tadpoles (Bogi et al., 2002; Kloas, 2002; Kloas and Lutz, 2006).

1.5.3. In bony fish

Endocrine disruption in fish by environmental estrogens and xenoestrogens is considered to be a major problem in fish populations (Jobling and Tyler, 2003a; Mills and Chichester, 2005). For example, some of the pioneering work of Sumpter and coworkers in the United Kingdom showed the presence of feminized male fish in rivers downstream of waste water treatment plants. The occurrence has been associated with estrogens and some types of industrial chemicals in the water such as alkylphenols (Purdom et al., 1994; Desbrow et al.,

1998; Routledge et al., 1998). Discharges from pulp and paper mill effluents also have been linked to alterations in endocrine function in fish, suggesting that plant byproducts can affect processes controlled by estrogens and androgens (Van der Kraak, 1998; Larsson et al., 1999; Park et al., 2001). Other examples of possible impairing effects of EDCs on fish populations have been related to tetrachlorodibenzodioxin and PCB in the Great Lakes, and polycyclic aromatic hydrocarbons at marine sites (Fairbrother et al., 1999). Recent research has demonstrated that many aquatic ecosystems receive inputs of estrogens, such as E2 and synthetic estrogens (Cargouet et al., 2004; Kolodziej et al., 2004).

The aquatic environment is a sink for chemical substances, and therefore, aquatic animals are particularly endangered by the action of EDCs (Tyler et al., 1998). For teleost fish, observations such as the appearance of VTG, an egg yolk precursor that is produced by oviparous female fish in response to circulating plasma estrogens, in male fish (Purdom et al., 1994; Allen et al., 1999) or gonadal and hormonal alterations (Jobling et al., 1998; Munkittrick et al., 1998; Bortone and Cody, 1999) have attracted attention to the contamination of the aquatic environment by EDCs and their potential impairing impact on wild fish populations (Jobling and Tyler, 2003). The original work conducted on freshwater fish in the UK established that treated sewage effluents were estrogenic and induced VTG in male individuals (Purdom et al., 1994).

The estrogen-active substances contained in the effluents include natural and synthetic hormones but also synthetic substances such as alkylphenols (Desbrow et al., 1998). In addition to the production of VTG, exposure to treated sewage effluents has been thought to cause pathological alterations of gonad morphology, such as formation of intersex gonads, because feminisation of reproductive ducts or oocytes in predominantly testicular tissue appeared. Intersex as a consequence of exposure to estrogenic effluents has been most intensively studied in the roach, *Rutilus rutilus* (Jobling et al., 1998), and evidence has been presented that the reproductive capacity of intersex roach is reduced (Jobling et al., 2002).

Any lipophilic contaminants in the water are readily absorbed. Because the detoxication capabilities of fish are less effective than those of mammals, the effects of EDCs are more apparent. Bioaccumulation also occurs, especially in bottom-feeding fish and those at the top of the food chain, because most EDCs are lipid-soluble and concentrated in fat tissues. Most studies available have been carried out on estrogen exposure, using the presence of VTG in the plasma of male fish as a biomarker for feminisation. A variety of effects of EDCs on fish populations have been reported. The domestic waste water effluent from sewage treatment works has been shown to be estrogenic, to cause hermaphroditism and

increase in plasma VTG in male fathead minnow (Hemming et al., 2001). This possibly reflects the presence of steroidal contraceptive agents such as EE2, which are not easily removed by conventional sewage treatments (D'Ascenzo et al., 2003; Cargouet et al., 2004). Similar effects have been reported for a wide range of fish species. In general, the experiments show that fish reproduction is very sensitive to environmental contamination, especially from sewage plants and industrial discharges (Arukwe, 2001).

Plasticisers are examples of environmental compounds, which affect fish reproduction. Alkylphenols such as nonyl- and octylphenol are weak estrogen mimics, and exposure of fish to these compounds has been linked with increased mortality rates, reduced reproductive capacity and VTG synthesis in male fish and yolk degeneration in oocytes (White et al., 1994). While the primary event of the estrogenic action of these compounds appears to be their binding to the ER, the mechanisms through which they disrupt physiological functions are not understood to date.

Fish are appropriate models for testing EDCs, not only from the perspective of existing ecological impacts, but also in terms of species extrapolation. Particulary, there is a significant degree of conservation of basic aspects of the hypothalamic-pituitary-gonadal axis across the vertebrates line of evolution (Ankley et al., 1997). This provides an excellent basis for using results from fish tests to predict likely mechanisms of action of potential EDCs in other vertebrate. Moreover, much of the basic molecular machinery involved in initiation of toxic responses is highly conserved across vertebrate species.

Different experimental approaches for partial- and full-life cycle tests with fish that enable the investigation of a broad range of EDCs have been described (Ankley and Johnson, 2004). In the United States, the Environmental Protection Agency (EPA) uses the fathead minnow, in a short-term (21-day) assay. Chemicals identified as potential EDCs in the short-term screening experiments might then be subjected to more intensive full-life cycle or even multigenerational assays with a number of vertebrate species. As model species fish such as the fathead minnow, Japanese medaka (*Oryzias latipes*), or sheepshead minnow (*Cyprinidon variegatus*) are included. From the international perspective, the OECD has formed a task group focused on developing internationally harmonised test methods for EDCs for both mammalian and nonmammalian species (Huet, 2000). A subcommittee within the task group is currently focusing specifically on fish tests. Three small fish species, i.e. fathead minnow, medaka, and zebrafish, are being evaluated for screening (partial-life cycle assays) as well as more extensive (full-life cycle) testing of EDCs (OECD, 2004).

Induction of VTG is used as a biomarker of exposure of fish to estrogenic substances, although standardised protocols are not yet available (Navas and Segner, 2006). Besides VTG there are also other biomarkers such as secondary sexual characteristics, gonadosomatic indices (GSI), plasma steroids, gonadal histology, enzyme induction, and gene expression. Hutchinson and colleagues (2006) consider that biomarker (nonadverse) signals provide mechanistic signals to guide chronic testing for adverse effects. At present, they are not recommended to be used to derive PNEC (predicted no-effect concentrations) values for EDCs. Adverse effects (survival, length, weight, development, fecundity fertilisation rate, hatching success) measure population-relevant parameters and should be used for calculating PNEC values for EDCs (Hutchinson et al., 2006).

1.6. The IGF-system in bony fish

In mammals, the insulin-like growth factor (IGF) system consists of IGF-I and IGF-II, cell surface receptors IGF-I (IGF-1R) and IGF-II (M-6-PR), high affinity binding proteins (IGFBP-1 to -6), IGFBP cleaving proteases, an acid labile subunit (ALS) which is a liver-derived, growth hormone (GH)-regulated glycoprotein (Baxter and Martin, 1989), as well as several low-affinity IGFBP-related proteins.

It has been recognized that the GH/IGF-I axis plays an integral role in the neuroendocrine regulation of vertebrate growth. As outlined in the "somatomedin hypothesis", tissue secretions of IGF-I mediate the growth-promoting actions of pituitary GH by endocrine, paracrine, and/or autocrine mechanisms (Le Roith et al., 2001). At the cellular level, IGF-I triggers a variety of biological responses, such as proliferation, survival, migration, and differentiation. At the whole animal level IGF-I coordinates different cellular responses among the different tissues and cell types and regulates the pattern of growth.

The existence of IGF-I signalling in fish was uncertain until the late 1980s. IGF-I activity was detected in the serum of both teleost and elasmobranchs, using a porcine cartilage sulfation bioassay (Shapiro and Pimstone, 1977). Duan and Hirano (Duan and Hirano, 1990) have demonstrated that human IGF-I could stimulate sulphate uptake in cultured explants of eel gill arch, and have reported a GH-dependent sulfation factor in the serum of the Japanese eel (*Anguilla japonica*) (Duan and Inui, 1990). Evidence for a functional IGF-I system (ligand, receptor and IGFBPs) in all known vertebrate groups indicates its long evolutionary history (Duan, 1998; Plisetskaya, 1998; Reinecke and Collet, 1998).

Vertebrate IGFs (IGF-I and IGF-II) are members of the insulin superfamily, that is characterized by a common "insulin-like" tertiary structure, conserved cystein residues and a hydrophobic core (Chan and Steiner, 2000). To date, IGF-like molecules have not been identified in any invertebrates, whereas distinct insulin and insulin-like genes have been shown to exist in urochordates (McRory and Sherwood, 1997; Reinecke et al., 1999), suggesting that the original "IGF-like" gene diverged from insulin-like ancestor after divergence of the protochordates from their invertebrate ancestor. The IGF-1R and the insulin receptor (InsR) likely evolved from a common ancestral gene (Wood et al., 2005). The structural similarities between the InsR, IGF-1R and their ligands provide the possibility for heterologous ligand-receptor interactions (Nakae et al., 2001). This potential cross activation has been proposed to explain the origin and evolution of the IGFBPs in vertebrates that exhibit a high affinity for IGFs, but little affinity for insulin (Kelley et al., 2002;2006). In addition, some IGFBPs seem to have evolved additional ligand-independent functions in mammals (Duan, 2002).

1.6.1. Primary structure and mRNAs

IGF-I gene sequences have been characterized in diverse teleost species, i.e. tilapia, *Oreochromis mossambicus* (Reinecke et al., 1997), the scorpaeniform daddy sculpin, *Cottus scorpius* (Loffing-Cueni et al., 1998), the salmonids coho salmon, *Oncorhynchus kisutch* (Cao et al., 1989; Duguay et al., 1992), rainbow trout, *Oncorhynchus mykiss* (Shamblott and Chen, 1992), and chinook salmon, *Oncorhynchus tshawytschwa* (Wallis and Devlin, 1993), Japanese flounder, *Paralychthys olivaceus* (Tanaka et al., 1998), catfish, *Clarias macrocephalus* (McRory and Sherwood, 1997), goldfish, *Carassius auratus* (Kermouni et al., 1998), gilthead seabream, *Sparus aurata* (Duguay et al., 1996), Japanese eel, *Anguilla japonica* (Yamaguchi et al., 1999) and barramundi, *Lates calcarifer* (Kinhult, 1994).

Teleost IGF-I cDNA sequences encoded preprohormones range between 159 and 193 amino acids in length. All contain a putative signal peptide that is removed during secretion to yield the IGF-I prohormone of five domains (E-B-C-A-D) as also present in mammalian IGF-I. The mature IGF-I peptide (domains B-C-A-D), contains 68 or 70 amino acids, depending on the species. Overall, mature teleost IGF-I peptides exhibit 72-81% identity to human IGF-I, confirming that the IGF-I peptide has been well conserved throughout vertebrate evolution.

1.6.2. Expression patterns of IGF-I

Evidence from molecular studies on fish indicates that the IGF-I gene is transcriptionally active throughout all stages of fish development, including embryogenesis. IGF-I mRNA expression has been demonstrated in unfertilized eggs, fertilized embryos, and larvae of gilthead seabream (Duguay et al., 1996), zebrafish (Maures et al., 2002), and rainbow trout (Gabillard et al., 2003a), and in larva of barramundi (Stahlbom et al., 1999).

A number of studies have demonstrated that IGF-I mRNA is expressed in multiple tissues of juvenile salmonids, including muscle, spleen, fat, intestine, liver, heart, testes, ovary, kidney, pituitary, and brain (Duguay et al., 1992; Shamblott and Chen, 1993; Biga et al., 2004). IGF-I mRNA expression has also been demonstrated in hepatic and nonhepatic tissues of adult teleosts, including several salmonids (Duguay et al., 1992; Duan et al., 1993a; Sakamoto and Hirano, 1993; Duguay et al., 1994) daddy sculpin (Loffing-Cueni et al., 1998), tilapia (Reinecke et al., 1997; Caelers et al., 2004). Schmid et al. (1999) investigated the expression of IGF-I mRNA in tilapia ovary; using *in situ* hybridisation, they demonstrated a moderate expression of IGF-I mRNA in the ooplasm of young oocytes, and higher levels of expression in somatic follicle cells (granulosa and theca) of oocytes at the yolk globule stage. IGF-I was immunocytochemically detected in various tissues of larva and/or juvenile shi drum (Radaelli et al., 2003), turbot (Berwert et al., 1995), and gilthead seabream (Perrot et al., 1999). IGF-I has also been described in multiple tissues of adult tilapia and during early development, including somatic and germ cells of the gonads, in pancreatic endocrine cells, gastro-entero-endocrine cells, renal proximal tubule cells, interrenal cells, gill chondrocytes and chloride cells, skeletal muscles, cardiomyocytes, neurones (Reinecke et al., 1997; Schmid et al., 1999; Berishvili et al., 2006a; Berishvili et al., 2006b). Furthermore, IGF-I mRNA and peptide were located to subpopulations of endocrine cells in the adenohypophysis in developing and adult tilapia (Berishvili et al., 2006b; Eppler et al., 2007).

1.6.3. Regulation of IGF-I expression

There is abundant evidence that GH is the primary regulator of IGF-I synthesis and/or secretion in juvenile and adult fish. For example, GH injections have been shown to increase the hepatic IGF-I mRNA level in coho salmon (Cao et al., 1989; Duguay et al., 1994), rainbow trout (Sakamoto and Hirano, 1993; Shamblott et al., 1995), gilthead seabream (Duguay et al., 1996), tilapia (Shepherd et al., 1997), zebrafish (Maures et al., 2002) and carp

(Hashimoto et al., 1997). Plasma or serum levels of IGF-I and/or tissue responsiveness to IGF-I, have been demonstrated to be GH-dependent in a number of teleost species, including tilapia (Shepherd et al., 1997; Kajimura et al., 2001), eel (Duan and Hirano, 1992) and salmonids (Niu et al., 1993; Moriyama et al., 1994). Binding studies have confirmed the presence of high-affinity GH-binding sites in teleost liver (Shepherd et al., 1997). The existence of a functional (bidirectional) GH/IGF-I axis in fish is further supported by a number of *in vivo* and *in vitro* studies (Reinecke, 2006, compare Fig. 4). For example, GH stimulation of IGF-I mRNA expression has been shown to be dose-dependent in primary hepatocyte culture of coho salmon (Duan et al., 1993b; Pierce et al., 2004), rainbow trout (Shamblott et al., 1995) and tilapia (Schmid et al., 2000). Conversely, administration of IGF-I suppressed GH secretion in rainbow trout *in vivo* (Blaise et al., 1995), and from cultured tilapia pituitaries (Kajimura et al., 2002).

Although most studies have focused on GH regulation of hepatic IGF-I expression, the stimulatory effects of GH are not only limited to the liver. GH injection has been shown to increase also the IGF-I mRNA levels in retina, brain, gill, and intestine of goldfish (Otteson et al., 2002; Vong et al., 2003), in gill and kidney of rainbow trout (Sakamoto and Hirano, 1993), and in tilapia muscle (Kajimura et al., 2001). In agreement, high-affinity, saturable GH-binding sites have been identified in many nonhepatic tissues (Yao et al., 1991).

1.6.4. IGF-I receptors (IGF-1R) and IGF-binding proteins (IGF-BPs)

Full-length cDNA sequences encoding the IGF-1R have been reported in turbot (Elies et al., 1999), zebrafish (Maures et al., 2002), and Japanese flounder (Nakao et al., 2002). In addition, partial IGF-1R cDNAs have been published for seabream (Perrot et al., 1999), rainbow trout (Greene and Chen, 1999), goldfish (Otteson et al., 2002) and Arctic charr (Tao and Boulding, 2003).

The signal transduction pathways mediating IGF signalling through the IGF-1R include tyrosine kinase activity. The tyrosine kinase domains of both IGF-1Rs contain a number of conserved functional elements, including a putative ATP-binding site, a cluster of three tyrosine residues corresponding to the tyrosine phosphorylation site in the mammalian IGF-1R known to be required for IGF-1R signalling in mammals. The biological effects of IGF-1R activation are transduced by MAPK and PI3-kinase transduction pathways (Wood et al., 2005). IGF-1R mRNA is widely expressed among different fish species. The nearly ubiquitous distribution of IGF-1R mRNA was demonstrated in embryonic and adult zebrafish

(Ayaso et al., 2002; Maures et al., 2002), rainbow trout (Greene and Chen, 1999; Gabillard et al., 2003; Biga et al., 2004), seabream (Perrot et al., 1999), goldfish (Otteson et al., 2002), and Japanese flounder (Nakao et al., 2002). IGF-I binding was detected in retina, skeletal and cardiac muscle, ovary and brain of several species (Gutierrez et al., 1993; Blaise et al., 1995; Párrizas et al., 1995; Moon et al., 1996).

Proteins with binding affinity and specificity for the IGFs have been identified in the serum of multiple teleosts (Degger et al., 2000; Kajimura et al., 2003; Shimizu et al., 2003a; Shimizu et al., 2003b; Beckman et al., 2004). In most fish species studied, IGF-I is observed to bind most abundantly to a protein of 40-45 kDa (Shimizu et al., 1999), similar in size to the IGFBP-3 to which the majority of IGFs are bound in mammals. Other IGFBPs detected in fish serum also exhibit similarities in size to mammalian IGFBPs, such as IGFBP-1 (29-31 kDa), IGFBP-2 (30-32 kDa), and IGFBP-4 (21-24 kDa) (Kelley et al., 2006).

1.7. Biological actions of the IGF system in fish

The critical role of IGF-I as a primary regulator of somatic growth in fish is well illustrated by the stunted growth model. Transfer of yearling salmon from freshwater to seawater before physiological completion of smoltification results in significant growth retardation (Wood et al., 2005). Stunted fish exhibited elevated levels of serum GH when compared with normally growing fish. The reduced expression of hepatic IGF-I mRNA was associated with a reduced level of circulating IGF-I protein (Duan et al., 1995). IGFBP-3 was increased under anabolic circumstances, whereas lower molecular weight IGFBPs (IGFBP-1, -2, -4) were upregulated in catabolic phases, e.g. fasting (Kelley et al., 2001). The homolog to mammalian IGFBP-3 appears to facilitate IGF-I signalling in teleost, perhaps by prolonging the half-life of IGF-I, while lower molecular weight IGFBPs are thought to suppress IGF-I-mediated growth by inhibiting activation of the IGF-1R.

Human IGF-I and recombinant teleost IGF-I peptide exhibited equal potency in stimulating sulfation activity in cartilage explants of salmon and seabream (Fine et al., 1997). Similar effects have been reported in other teleost fish, including tilapia (Ng et al., 2001), carp (Cheng and Chen, 1995), and rainbow trout (Takagi and Bjornsson, 1996). IGF-I has potent mitogenic effects in a variety of tissues and cell types. For example, recombinant human IGF-I potently stimulated [^{3}H]thymidine incorporation into eel cartilage (Duan and Hirano, 1992), rainbow trout myoblasts, spermatogonia, and spermatocytes (Loir and Le Gac, 1994), and

zebrafish embryonic cells (Duan et al., 1999). There is abundant evidence that IGF-I stimulates protein synthesis in fish organs. For example, recombinant human IGF-I stimulated L-alanine uptake in rainbow trout myocytes (Castillo et al., 2004), and both L-alanine uptake and L-leucine incorporation in brown trout cardiomyocytes (Gallardo et al., 2001).

There is evidence suggesting the functional impact of IGF signalling during early development in fish. For example, coexpression of mRNAs encoding IGF ligands and receptors has been reported in zebrafish embryos, and also the presence of proteins corresponding to functional IGF receptors (Maures et al., 2002). Our group has investigated the cellular sites of IGF-I mRNA and peptide synthesis in the early developing tilapia (0-140 days post fertilization, DPF). IGF-I appeared early (4-5 DPF), first in liver, the main source of endocrine IGF-I, and then in organs involved in growth or metabolism, i.e. liver, exocrine and endocrine pancreas, chondrocytes, skeletal muscle cells, neurohypophysis and adenohypophysis (Berishvili et al., 2006b). The expression of IGF-I was more pronounced during development than in juvenile and adult life. Local IGF-I therefore seems to have a high functional impact in early growth, metabolism and organogenesis. Targeted knockdown of the duplicate IGF-1Rs in zebrafish embryos by morpholino-modified oligonucleotides resulted in dramatic developmental perturbations and even death, indicating distinct requirements for IGF signalling during embryonic development (Schlueter et al., 2003).

The GH/IGF-I system has been long recognized as an important participant in the osmoregulatory physiology of fish. McCormick and colleagues were among the first to demonstrate that exogenous administration of IGF-I to salmonids improved their ability to maintain plasma osmolality and sodium levels during seawater challenge (McCormick et al., 1991). Subsequent studies showed that IGF-I stimulated gill Na^+, K^+,-ATPase activity, indicating a direct effect of IGF-I on osmoregulation in salmonids (Madsen and Bern, 1993). Transfer of seawater-adapted tilapia to freshwater resulted in reduced growth and decreased tissue levels of GH and IGF-I mRNA, but increased serum levels of GH and IGF-I, suggesting that osmoregulatory stress may directly activate the GH/IGF-I axis without any concomitant stimulation of somatic growth (Riley et al., 2003).

Fish are convenient models to investigate gonadal development and function, and studies have indicated an important role of the IGF-I system in fish reproductive development. Fish gonads express mRNAs encoding IGFs (Duguay et al., 1992; Duan and Plisetskaya, 1993; Duguay et al., 1994; Perrot et al., 2000; Berishvili et al., 2006a), the IGF-1R (Perrot et al., 2000; Maures et al., 2002), and IGFBPs (Funkenstein et al., 2002). IGF-I has been localized in the ovary of tilapia (Schmid et al., 1999), carp (Gutierrez et al., 1993), and

in rainbow trout spermatogonia and spermatocytes (Loir and Le Gac, 1994). IGF-I signalling has been shown to influence the synthesis and/or secretion of reproductive steroid hormones from the fish gonadal tissues. For example, both human and recombinant salmon IGF-I have been shown to inhibit basal and LH-stimulated testosterone and 17α-hydroxyprogesterone production by isolated theca-interstitial cells from coho salmon preovulatory follicles. In addition, IGF-I stimulated production of 17β-estradiol and 17,20β-progesterone (17,20 β-P) by granulosa cells from prematurational follicles, and of 17,20 β-P by granulosa cells from maturational follicles (Maestro et al., 1997). Recombinant IGF-I alone had no effect on spermatogenesis by Japanese eel testicular fragments, but enhanced the stimulatory effects of 11-ketotestosterone (Nader et al., 1999).

1.8. Influence of endocrine disruptors on the fish IGF system

Thus, IGF-I plays a central role in the complex system that regulates fish growth, differentiation, and reproduction. It selectively promotes mitogenesis and differentiation and inhibits apoptosis (Jones and Clemmons, 1995; Reinecke and Collet, 1998). Among the non-mammalian classes, bony fish are the mostly studied with respect to IGF-I (Duan, 1998; Plisetskaya, 1998; Reinecke et al., 2005; Wood et al., 2005) mainly due to their unique development from the larval to the adult life and to their high importance in aquaculture. Thus, there is rising interest in the significance of IGF-I in fish development, growth and reproduction. Some evidence has been presented (Riley et al., 2004; Carnevali et al., 2005; McCormick et al., 2005) that estrogens may influence acutely synthesis and/or release of IGF-I from adult fish liver and affect sulfate uptake into cultured cartilage (Ng et al., 2001). Very recently, the influence of exposure to E2 on several genes, including IGF-I, has been investigated in an adult cyprinid, the fathead minnow (Filby et al., 2006). Thus, there is some evidence that organisational effects of estrogens on growth and reproduction of fish may be mediated via IGF-I. These effects may be even more pronounced during fish development, the phase of most rapid growth, sexual differentiation and gonad development.

A direct effect of BPA on osteoclasts and osteoblasts has been shown in a culture system of goldfish scales (Suzuki and Hattori, 2003), using tartrate-resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) as markers for osteoclasts and osteoblasts, respectively. BPA at 10^{-5} M significantly suppressed both TRAP and ALP activities *in vitro* and *in vivo* and decreased the expression of IGF-I mRNA. However, E2 stimulated TRAP and

ALP activities and did not alter IGF-I mRNA expression, suggesting that BPA and E2 may differently affect bone metabolism (Suzuki and Hattori, 2003). Furthermore, BPA more pronouncedly interacted with local IGF-I in bone than E2.

A further physiological process in fish where estrogens and xenoestrogens may interfere with the GH/IGF-I system is smoltification, i.e. the developmental stage through which the stream-dwelling parr is transformed into the seaward migrating smolt. Here GH plays a central role likely both by direct and indirect action, the latter being mediated via endocrine or local IGF-I on the expression of gill Na^+, K^+-ATPase. Recent evidence indicates that the role of IGF-I in smoltification can be modulated by estrogens. E2 (100 ng/L) and 4-nonylphenol (20 μg/L) significantly reduced plasma IGF-I by about 25% in Atlantic salmon during smoltification (Arsenault et al. 2004), and it has been assumed that environmental xenoestrogens such as 4-nonylphenol have contributed to the historical decline of anadromous salmons in Eastern Canada (Fairchild et al., 1999).

Thus, to date only few studies have addressed the question of possible interferences between EDCs and the IGF-I system of fish. Provided that the preliminary data are strengthened by further studies, E2 and estrogenic compounds must be considered to have a higher impact as endocrine disruptors as yet thought because they interfere with a key hormonal system that is involved in numerous processes of metabolism, reproduction, differentiation, growth, development and ageing.

1.9. Aim of the study

The present study aims to investigate the potential influence of EE2, as representative potent estrogen, on IGF-I when applied to developing male and female fish.

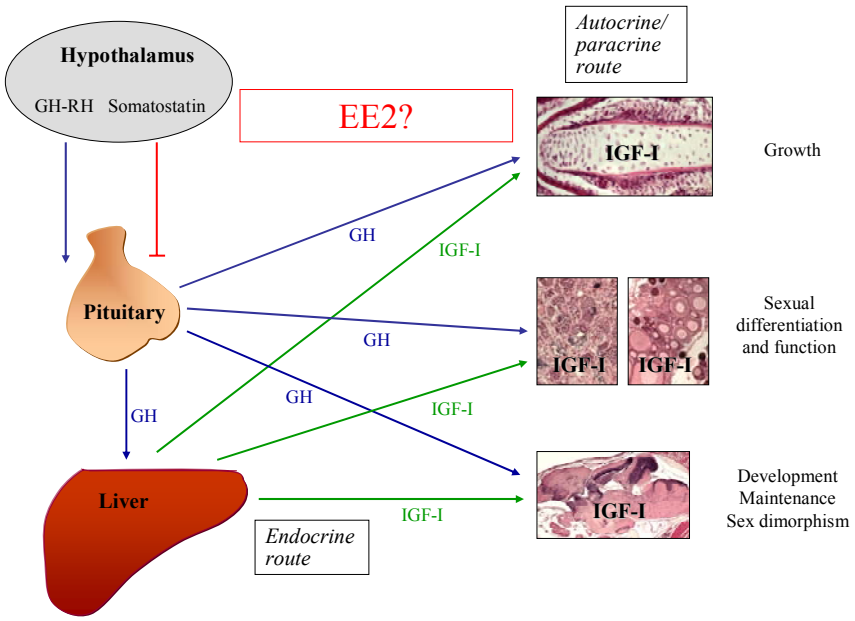

Fig. 4 The GH-IGF-I axis in teleost fish (Scheme modified from Reinecke, 2006).

For that purpose, two experimental approaches were selected:

1. A balanced population of tilapia (*Oreochromis niloticus*) was fed during 10-40 DPF with EE2 at the optimal dosage to induce functional feminisation. This period represents the sensitive window of gonad differentiation in tilapia (Fig. 5). We assessed whether EE2 treatment during this development stage exerts lasting effects on gonad differentiation, growth, ER expression and crucial issues of the GH/IGF-I system. The following parameters were determined after termination of EE2 treatment at 50, 75, 90 and 165 DPF: sex ratio, growth, serum IGF-I levels, and mRNA expression levels of IGF-I, GH and ERα. Furthermore, we studied the potential alterations of IGF-I mRNA expression in the organ-specific cells of the organs investigated after EE2 treatment by in situ hybridisation.

The target organs studied included those of the central GH/IGF-I axis, i.e. pituitary and liver. Although fish liver is the major source of circulating IGF-I, IGF-I also occurs in extrahepatic sites (Reinecke et al., 1997) where it is particularly expressed during

development (Duguay et al., 1996; Perrot et al., 1999; Radaelli et al., 2003; Berishvili et al., 2006b). Thus, the alterations in IGF-I mRNA expression were also determined in organs showing high IGF-I expression during ontogeny, i.e. gills and brain. We further examined the influence of EE2 exposure on ERα and IGF-I expression in the gonads. Gonad differentiation of developing fish can be altered by estrogen treatment (Segner et al., 2006), and gonads of developing fish express IGF-I mRNA (Berishvili et al., 2006a), although the functional role of IGF-I expression in the gonad, and its response to estrogen exposure are unknown yet.

2. In a second approach, developing fish were exposed to environmental concentrations of EE2 starting from 10 DPF until end of experiment (100 DPF). This period includes the sensitive window of gonad differentiation in tilapia (Fig. 6). We assessed whether EE2 treatment at environmental concentrations exerts effects on gonad differentiation, growth, ER expression and crucial issues of the GH/IGF-I system. The following parameters were determined at 30, 50, 75 and 100 DPF: sex ratio, growth and mRNA expression levels of IGF-I, and ERα in liver, gonads, gills and brain.

2. Material and Methods

2.1. Production and maintenance of fish

The *Oreochromis niloticus* fry, used in this study, originated from the Aquaculture Experimental Facilities of CIRAD (Montpellier). Fertilized eggs were obtained through either natural or artificial fertilisation (Berishvili et al., 2006a).

For natural fertilisation, *O. niloticus* breeders (1 male and 3 females) were maintained in a spawning aquarium of 360 L maintained at constant temperature (27°C) and photoperiod (12light:12dark). Under natural photoperiods, reproductive activity occurs mainly from the afternoon until sunset in these species (Baroiller et al., 1997). Consequently, we checked every morning for the presence of newly incubating females, which were then isolated from the other fish. On the first day after fertilization, eggs were gently removed from the mouth of the female and incubated in 1L McDonald jars at 27±1°C (mean range).

For artificial fertilisation, the maturation of isolated females was carefully recorded (i.e. uro-genital papilla development and behaviour). When the near-breeding stage was reached, both male and female breeders were striped under anaesthesia. Fertilisation was performed according to (Chourrout and Itskovich, 1987). After fertilisation, eggs were incubated in 1L McDonald jars, maintained at 27°C, to the free-swimming stage (around 10 DPF).

2.2. Hormone treatment with high concentration of EE2

Just before the completion of yolk sac absorption, fry were placed into a 40L tank in an indoor recirculating system at 27 ± 1°C. Three natural populations (n=200 each) of tilapia were fed in September during a period covering the sensitive period, i. e. 10-40 DPF with EE2 at the optimal dosage (125 µg EE2/g food) to induce functional feminisation in most individuals. Hormonally treated commercial salmonids food was prepared by the ethanol evaporation method (0.6 L of 95% ethanol/kg food). EE2 (Sigma E-4876, Switzerland) was dissolved in 95% ethanol and the solution sprayed over the food (125 µg EE2/g food). Control food was prepared in the same way without EE2. After ethanol evaporation, the food was stored at 4°C. Fry were fed at 20% of their biomass per day for the first week, 15% for the second week, 12%

for the third week and 10% for the last week. They were fed six times daily. Starting from 41 DPF both populations were fed with normal salmonid food until sampling (Fig. 5).

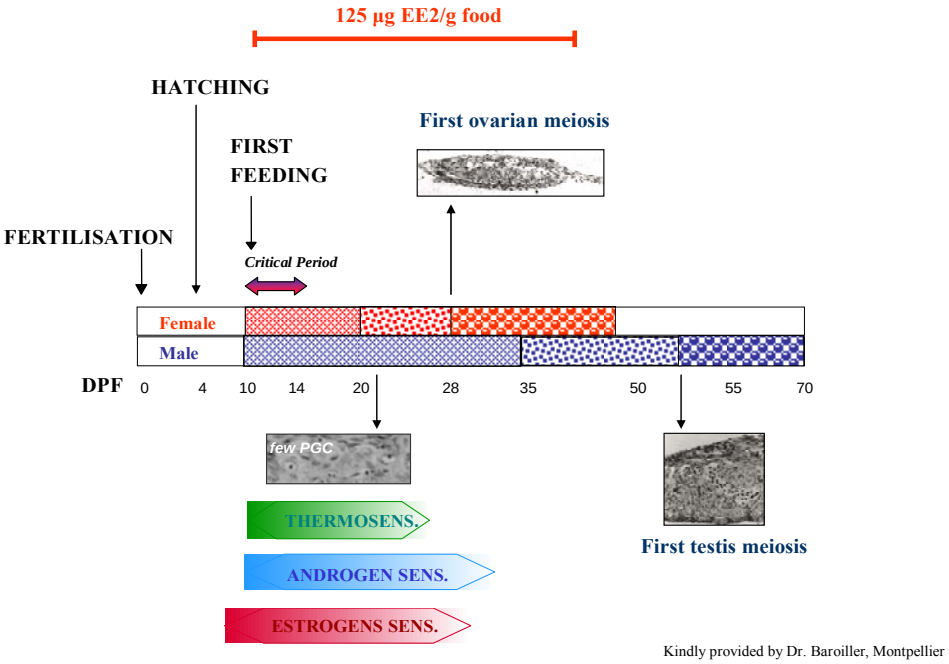

Fig. 5 Developmental stages of tilapia and hormone treatment with high concentration of EE2 (kindly provided by Dr. Baroiller, Montpellier).

At the age of 50, 75, 90 and 165 DPF, fish were sampled and those from 75, 90 and 165 DPF additionally used for histologic sex determination to evaluate statistically feminisation efficiency. Control (male: 50 DPF n=12, 75 DPF n=9, 90 DPF n=9, 165 DPF n=9; female: 50 DPF n=15, 75 DPF n=12, 90 DPF n=14, 165 DPF n=12) and EE2-treated (male: 50 DPF n=12, 75 DPF n=10, 90 DPF n=12, 165 DPF n=11, female: 50 DPF n=12, 75 DPF n=12, 90 DPF n=14, 165 DPF n=11) tilapia were sampled. Blood samples were obtained from control (male: 75 DPF n=7, 90 DPF n=6, 165 DPF n=9; female: 75 DPF n=7, 90 DPF n=7, 165 DPF n=12) and EE2-treated (male: 75 DPF n=7, 90 DPF n=6, 165 DPF n=7; female: 75 DPF n=8, 90 DPF n=8, 165 DPF n=9) tilapia.

2.3. Hormone treatment with environmental concentrations

Two monosex populations XX and XY (n=300 each) of tilapia were artificially fertilised. In our study, we have used so-called monosex populations of tilapia. As histologically assessed

during the sampling procedure, the percentage of females in the female monosex group amounted to about 76% and that of males in the male monosex group to 80%. A population with 75% or more fish of the same sex is considered to be monosex (D'Cotta et al., 2001). Thus, our percentage is well within the range that can be achieved in tilapia (Baroiller et al., 1995) because sex chromosome–linked genetical factors determine sex largely but not exclusively, and the development of final sex phenotype is influenced by autosomal genetic factors in combination with the hormonal, behavioral, and environmental cues (Baroiller et al., 1999; Devlin and Nagahama 2002). For experimentation fish were transferred to the aquaria from 1L McDonald jars around 10 DPF just before the completion of yolk sac absorption and incubated in 40L aquaria at 27 ± 1°C during a period of 10-100 DPF at the concentrations 5 ng/L EE2, 25 ng/L EE2 and solvent control, respectively. Each group was run in duplicate aquaria, saturated prior treatment experiment with EE2 at concentration of 1000 ng/L during one week. Fish were fed with commercial salmonid food at 20% of their biomass per day for the first week, 15% for the second week, 12% for the third week and 10% for the last week. They were fed six times daily.

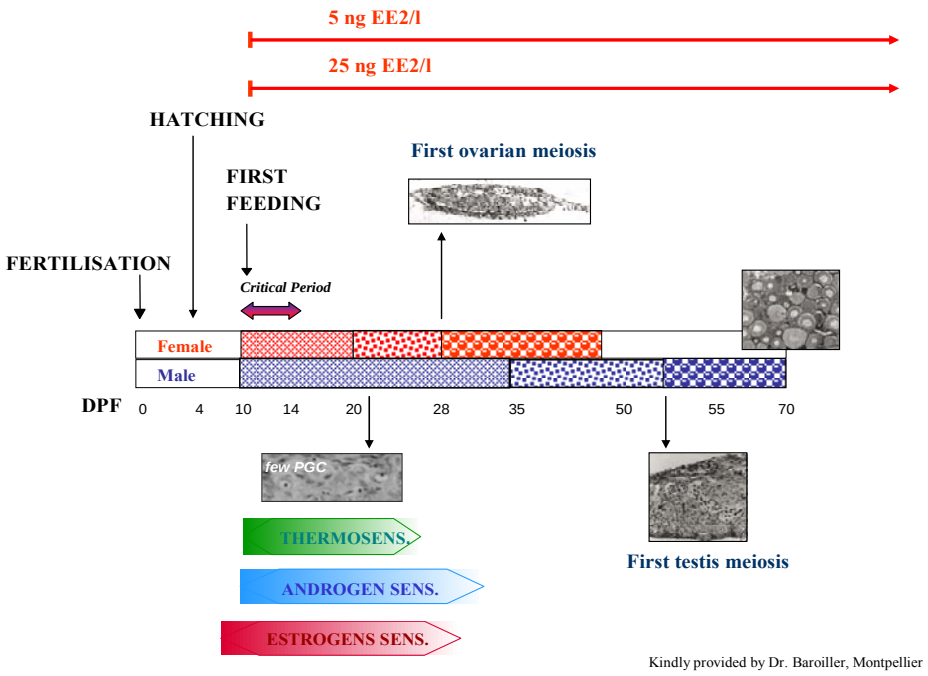

Fig. 6. Developmental stages of tilapia and treatment with environmental concentrations of EE2 (kindly provided by Dr. Baroiller, Montpellier).

At the age of 30, 50, 75 and 100 DPF fish were sampled and those from 75, 90 DPF additionally used for sexing statistics to evaluate feminisation efficiency. Control (male: 30

DPF n=8, 50 DPF n=12, 75 DPF n=11, 100 DPF n=12; female: 30 DPF n=10, 50 DPF n=10, 75 DPF n=11, 100 DPF n=12) and EE2-exposed (5 ngEE2/L, male: 30 DPF n=8, 50 DPF n=8, 75 DPF n=7, 100 DPF n=8; female: 30 DPF n=9, 50 DPF n=8, 75 DPF n=10, 100 DPF n=8; 25 ngEE2/L, male: 30 DPF n=7, 50 DPF n=7, 75 DPF n=8, 100 DPF n=8; female: 30 DPF n=7, 50 DPF n=8, 75 DPF n=9, 100 DPF n=8).

2.4. Solid phase extraction/ELISA

Ethinylestradiol ELISA kit (Ecologiene, Japan, purchased from Biosense Laboratories, Norway) was used for EE2 detection in water samples. In order to concentrate EE2 content to the detectable limits of the assay, water samples were prepared by solid phase extraction (SPE), according to Ternes et al. (Ternes et al., 1999). For this purpose, 100 mg LiChrolut® EN and 200 mg RP-18 cartridges (Merck) were first conditioned with the series of passing - Hexane, Acetone, Methanol and water pH 3.0. Pre-filtered water samples (200 mL) were loaded under a gentle vacuum pressure (1L/1h). Loaded cartridges were dryed under a nitrogen flow for 45-60 min. Elution was performed with 4 mL aceton, in silanised amber vials (Supelco) to collect the eluent. Aceton was evaporated to dryness under a stream of nitrogen and EE2 was reconstituted in 0.5 mL ethanol. Resulting samples were analysed by ELISA. The procedure was done according to the manufacturer's instructions.

2.5. Fish tissue preparation

Fish were anaesthetised with 2-phenoxy-ethanol (Sigma) added to water and measured in weight and length. Blood was collected with one mL heparinised syringes, centrifuged for 15 min at 4°C at 10000 rpm, serum was collected and stored at -20°C until use. Tissue specimens (liver, brain, gonads, gills, pituitary) were excised and immediately transferred into 1.5 mL of RNAlater™ (Ambion, Austin, TX, USA), kept overnight at 4°C to allow RNAs inhibitors to act and lateron stored at -20°C until use. Specimens for in situ hybridisation were fixed in Bouin's solution without acetic acid for 4 h at room temperature, dehydrated in ascending series of ethanol and routinely embedded in paraplast (58°C).

2.6. Radioimmunoassay for IGF-I

Serum IGF-I levels were determined in undiluted samples by RIA after SepPak C18 chromatography (Waters Corp., Milford, MA, USA), as described earlier (Zapf et al., 2002). In brief, for determination of serum IGF-I, 0.15 mL PBS containing 0.2% human serum albumin (HSA), pH 7.4, were added to 0.1 mL serum. For determination of pancreatic IGF-I, 0.5 mL of the extract was lyophilised, dissolved in 0.25 mL of PBS/0.2% HSA and centrifuged. All samples were acid-treated and run over SepPak C18 cartridges according to the protocol supplied by Immunonuclear (Stillwater, MN, USA). After reconstitution with 1 mL PBS/0.2% HSA samples were assayed at three different dilutions (1:5, 1:10, 1:20). 0.2 mL of samples or standards (tilapia IGF-I from GroPep, Adelaide, Australia) and 0.1 mL of IGF-I antiserum (final dilution 1:20000) were preincubated for 24 h at 4°C. 25000-35000 cpm of ^{125}I-IGF-I (Anawa, Wangen, Switzerland, specific activity 300-400 μCi/μl) were added to the final incubation volume (0.4 mL). The reaction mixture was incubated for another 24 h before precipitation with goat anti-rabbit gammaglobulin antiserum. After centrifugation, the pellet was counted in a gamma-counter.

2.7. Design of primers and probes for real-time PCR

Based on the mRNA sequences of *Oreochromis mossambicus* IGF-I (Reinecke et al., 1997), and *O. niloticus* β-actin as a a house-keeping gene (Hwang et al., 2003) primers (ß-actin: sense GCCCCACCTGAGCGTAAATA, antisense AAAGGTGGACAGGAGGCCA; IGF-I: sense TCTGTGGAGAGCGAGGCTTT, antisense CACGTGACCGCCTTGCA) and probes (ß-actin: TCCGTCTGGATCGGAGGCTTCATC; IGF-I: ATTTCAATAAACCAACAGGCTATGGCCCCA) were created as already described (Caelers et al., 2004). Using this method new primers and probes for GH (Caelers et al., 2005) and ERα (sense CAAGTGGTGGAGGAGGAAGATC, antisense CTCAGCACCCTGGAGCAG, probe CTGATCAGGTGCTCCTC) based on *O. niloticus* GH (Ber and Daniel, 1992) and ERα sequences (Chang et al., 1999) were designed with Primer Express software version 1.5 (PE Biosystems, Foster City, CA, USA). ERα was selected since it is considered to be the strongest indicator for response of the estrogen system to challenge with estrogen in teleosts (Filby and Tyler, 2005). The internal probes were labelled at the

5'end with the reporter dye 6-carboxyfluorescein (FAM), and at the 3'end with the quencher dye 6-carboxytetramethyl-rhodamine (TAMRA, both Eurogentec, Herstal, Belgium). Primer accuracy was verified by RT-PCR, the products were sequenced, and the integrity assured on a 2.5 % agarose gel.

2.8. Quantitation of IGF-I, GH, and ER-α expression by two-step real-time RT-PCR TaqMan system

Total RNA was extracted from specimens stored in 1.5 mL of RNAlater™ using TRIzol™ reagent (Invitrogen, Merelbeke, Belgium) and treated with 1 U of RQ1 RNAse-free DNAse (Catalys AG, Wallisellen, Switzerland). cDNA was synthesized from 800 ng of total RNA using 1 x TaqMan® RT Buffer, MgCl2 (5.5 mM), 1.25 U/µl of MuLV reverse transcriptase, 2.5 µM of random hexamers primers, 0.4 U/µl ribonuclease inhibitor, 500 µM each dNTP (Applied Biosystems, Rotkreuz, Switzerland) for 10 min at 25˚C, 30 min at 48˚C and 5 min at 95˚C. 2µl of cDNA obtained from 10 ng/µl total RNA were subjected, in duplicates, to real-time PCR using a Absolute™QPCR low ROX Mix (ABgene, UK) including Thermo-Start® DNA Polymerase, 300 nM of each primer, 150 nM of the fluorogenic probe. Amplification was performed using a total reaction volume of 10 µl in a MicroAmp Fast Optical 96-well reaction plate (Applied Biosystem). Reactions were run on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with the following thermal conditions: 95˚C for 15 min for enzyme activation followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min.

2.9. Relative quantification of treatment effects using the ΔΔCT method

The comparative threshold cycle ($\Delta\Delta C_T$) method (Livak and Schmittgen, 2001) was used to calculate relative gene expression ratios between EE2-treated and control groups. Data were normalized to *β*-actin as the reference gene. Efficiency tests for *β*-actin and IGF-I assays (Caelers et al., 2004) and ERα assay (data not shown) permitted the accurate use of the $\Delta\Delta C_T$ method. Relative changes induced by EE2-feeding were calculated by the formula $2^{-\Delta\Delta C_T}$, with $\Delta\Delta C_T = \Delta C_T$ (treated group) – ΔC_T (untreated control), and $\Delta C_T = C_T$ (target gene) – C_T (reference gene). All data are expressed as *n*-fold changes of gene expression in the experimental group relative to the control group, displayed in the graphs as 2 log scale, a

common way of presenting qPCR data, (Dzidic et al., 2006). Statistical significance was calculated using the nonparametric Mann-Whitney rank sum test (Mann and Whitney, 1947), which was selected as suitable variance analysis test. Exact P value is calculated. Statistical analyses were performed with GraphPad Prism© 4.

2.10. Preparation of probes for in situ hybridisation

Probes used for in situ hybridisation were prepared as already described (Schmid et al., 1999; Berishvili et al., 2006a; Berishvili et al., 2006b). In brief, total RNA from tilapia liver was extracted by the phenol/chloroform method (Chomczynski and Sacchi, 1987) with the Ultraspec Extraction Kit (ams, Lugano, Switzerland). For cDNA synthesis 5 µg RNA were annealed with 1 µM of a poly(dT) primer (5' CCTGAATTCTAGAGCTCAT(dT17) 3') for 3 min at 70°C. The RNA/primer mix was incubated for 1 h at 37°C with 15 mM dNTPs and 10 U AMV-RTase (Pharmacia, Switzerland) in 1x reaction buffer (50 mM Tris-HCl/pH 8.3, 40 mM KCl, 6 mM $MgCl_2$). One µl cDNA was incubated with 1 µM of sense (5`-GTCTGTGGAGAGCGAGGCTTT-3`) and antisense (5'-AACCTTGGGTGCTCTTGGCATG-3`) primers corresponding to the B- and E- domain, 200 µM dNTPs, and 1U Taq-polymerase (Pharmacia) in 1x incubation buffer (10 mM Tris-HCl/pH 8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001 % gelatine). The amplification program was optimised for a Stratagene RoboCycler Gradient 40: 1 cycle 10 min at 94°C, 1 min at 59°C, 2 min at 72°C; 30 cycles 1 min at 94°C, 1 min at 59°C and 2 min at 72°C followed by final extension of 5 min at 72°C. PCR fragments were separated on a 2% agarose gel and eluted by the Gel Extraction Kit QIAquick (Qiagen, Switzerland). Thereafter, the PCR products were cloned in a pCR-Script SK(+) cloning vector using a kit (Stratagene, Heidelberg, Germany). Plasmids containing the gene sequence fragments were sequenced (Microsynth, Switzerland) and the sequences compared to database. The plasmids containing the specific inserts of IGF-I (207 bp) were used as templates for the synthesis of digoxigenin (DIG)-labelled RNA probes. Linearisation was performed with *EcoRI* for T3- and *NotI* for T7-polymerase-driven transcription. One µg of linearised plasmids was transcribed *in vitro* in the presence of DIG-UTP from T3 and T7 promotors to obtain antisense and sense probes. Integrity of probes and efficiency of labelling were confirmed by dot blot and gel electrophoresis including blotting and incubation with antibody.

2.11. In situ hybridisation protocol

Four µm sections on Super Frost Plus slides (Menzel-Gläser, Germany) were dewaxed in Xylol, rehydrated in descending series of ethanol (100%, 96%, 70%), and postfixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 1 x PBS. The following steps were carried out with diethyl pyrocarbonate-treated solutions in a humified chamber: to denaturate proteins, sections were digested with 0.02% proteinase K in 20 mM Tris-HCl/pH 7.4, 2 mM $CaCl_2$ for 10 min at 37°C and, to reduce background, treated with 1.5% triethanolamine and 0.25% acetic anhydride for 10 min at room temperature. Slides were incubated with 100 µl prehybridisation solution/section for 3 h at 54°C. Hybridisation was carried out overnight at 54°C with 50 µl of hybridisation buffer containing 200 ng of sense (negative control) or antisense probes previously denaturated for 5 min at 85°C. Slides were washed for 15 min at room temperature in 2 x SSC, and for 30 min at 54°C at descending concentrations of SSC (2 x, 1 x, 0.5 x, 0.2 x). Sections were incubated with alkaline phosphatase-coupled anti DIG antibody diluted 1:4000 in 1% blocking reagent in buffer P1 for 1 h at room temperature in the dark. After washing twice in buffer P1 for 15 min, sections were treated with buffer P3, 5 mM levamisole and NBT/BCIP stock solution. Colour development was performed overnight at room temperature and stopped by rinse of the slides in tap water for 15 min. Thereafter, sections were mounted with glycergel. Microscopy and photography were performed with a Zeiss Axioscope using the Axiovision 3.1 software (Zeiss, Zürich, Switzerland).

Specificity as previously demonstrated for adult tilapia male and female gonads (Schmid et al., 1999; Berishvili et al., 2006b) and liver (Schmid et al., 1999) was reassured on adjacent sections of 50 DPF male brain with IGF-I antisense (Fig. 7A) and sense (Fig. 7B) probes and gills.

In situ hybridisation revealed positive signals with the antisense probe whereas no signals were present in the negative controls.

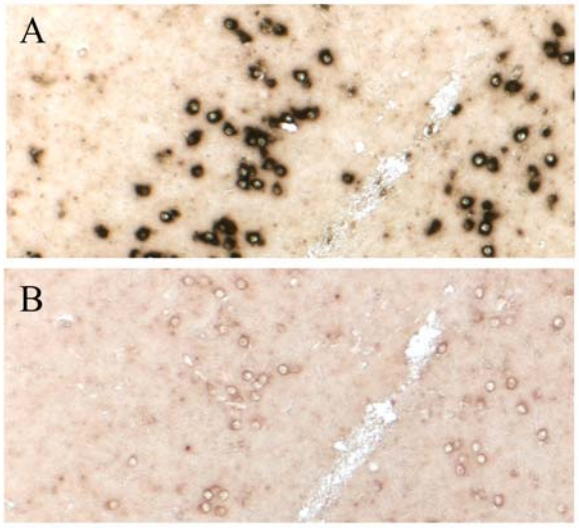

Fig. 7 Specificity of the in situ hybridisation technique shown on two adjacent sections of 50 DPF male tilapia brain hybridized with an IGF-I antisense probe (A) and an IGF-I sense probe (B).

3. Results

3.1. Treatment effects of EE2

Both, high-dose and environmental concentrations of EE2 strongly impacted the GH/IGF-I axis in tilapia and exerted persiting effects**.**

3.2. High dose EE2 treatment

Treatment of developing tilapia with high dose EE2 was performed as described (Shved et al., 2007).

3.2.1. Sex ratio

After EE2 treatment the sex ratio had shifted from 47.2 ± 8.5% females in control to 86.5 ± 14.1% at 165 DPF.

3.2.2. Body length and weight

EE2 treatment caused a progressive decrease in body length (BL) and body weight (BW) in both sex (Fig. 8). Both parameters were significantly lowered from 90 DPF onwards (90 DPF - BL: -16.9% in males, -13.4% in females; BW: -47.4% in males, -32.3% in females; 165 DPF - BL: -19.5% in males, -15.2% in females; BW: -46.2% in males, -40.1% in females).

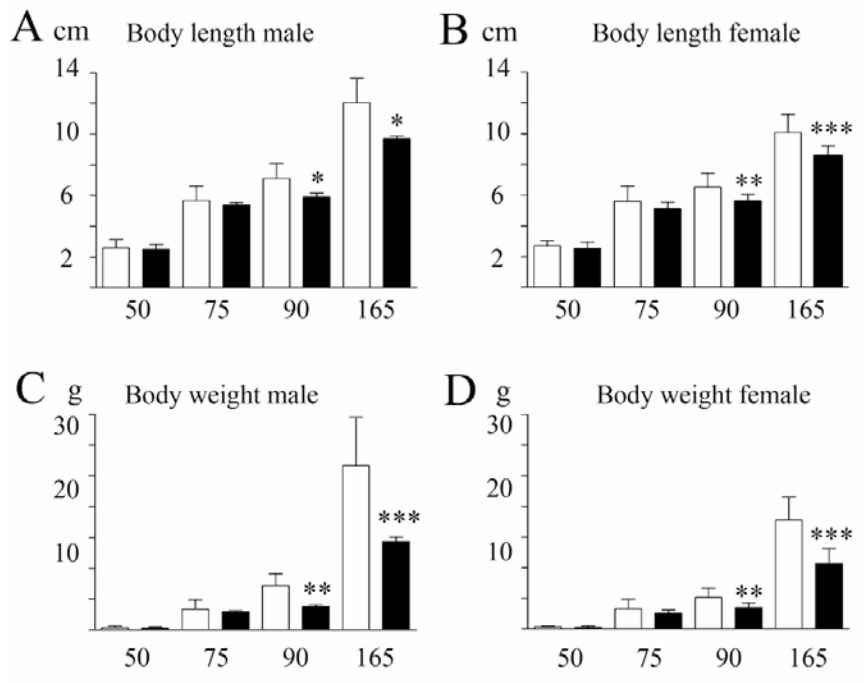

Fig. 8 Influence of EE2 exposure on fish growth throughout the experimental period. Body length (A,B, in cm) and weight (C,D, in g) in male (A,C) and female (B,D) control (white columns) and EE2-treated (black columns) tilapia. X-axis is labelled as DPF. Columns give mean values and bars SD. Significance levels: * P <0.01, ** P<0.005, *** P<0.0005.

3.2.3. Serum IGF-I level

While at 50 DPF the fish were too small to allow blood drawing blood could be taken at 75, 90 and 165 DPF. At 75 DPF, in males (Fig. 9G) the IGF-I serum level was significantly (P = 0.002) decreased in the EE2-treated group (5.65 ± 1.18 ng/mL) when compared to the controls (9.70 ± 2.06 ng/mL) while in females (Fig. 9H) there was only a trend to reduce IGF-I serum level (control 11.20 ± 2.18 vs. EE2-treated 8.54 ± 2.26, P=0.08). At the later stages, there was no significant difference between the treated group and the controls (Fig. 9G,H).

3.2.4. IGF-I mRNA and ERα mRNA levels in liver

Hepatic IGF-I mRNA was significantly reduced by EE2-feeding with the effect becoming evident in females (Fig. 9C) later than in males (Fig. 9A). At 50 DPF, IGF-I mRNA in liver was lowered in males by the 7.1-fold (P=0.05), at 75 DPF in males by the 3-fold (P=0.01) and in females by the 2.7-fold (P=0.002), at 90 DPF in males by the 1.7-fold (P=0.02) and in

females by the 5-fold (P=0.0003) and almost recovered in both sex at 165 DPF (Fig. 9A,C). In situ hybridisation revealed a markedly reduced number of hepatocytes containing IGF-I mRNA after EE2 treatment at 50 (Figs. 9E,F) and 75 DPF in male liver and at 75 and 90 DPF in female liver. Hepatic ERα mRNA was significantly increased after EE2-feeding to the 6.1-fold in males (P=0.01) and the 2.6-fold (P=0.004) in females at 50 DPF (Fig. 9B,D). At 75 DPF, ERα mRNA was raised to the 2.2-fold in males (P=0.02) and the 37-fold in females (P=0.002). In male liver, ERα was back at the normal level at 90 DPF. In female liver, ERα at 90 DPF was still raised to the 2.5-fold (P=0.05) of the control mRNA and at about the normal level at 165 DPF.

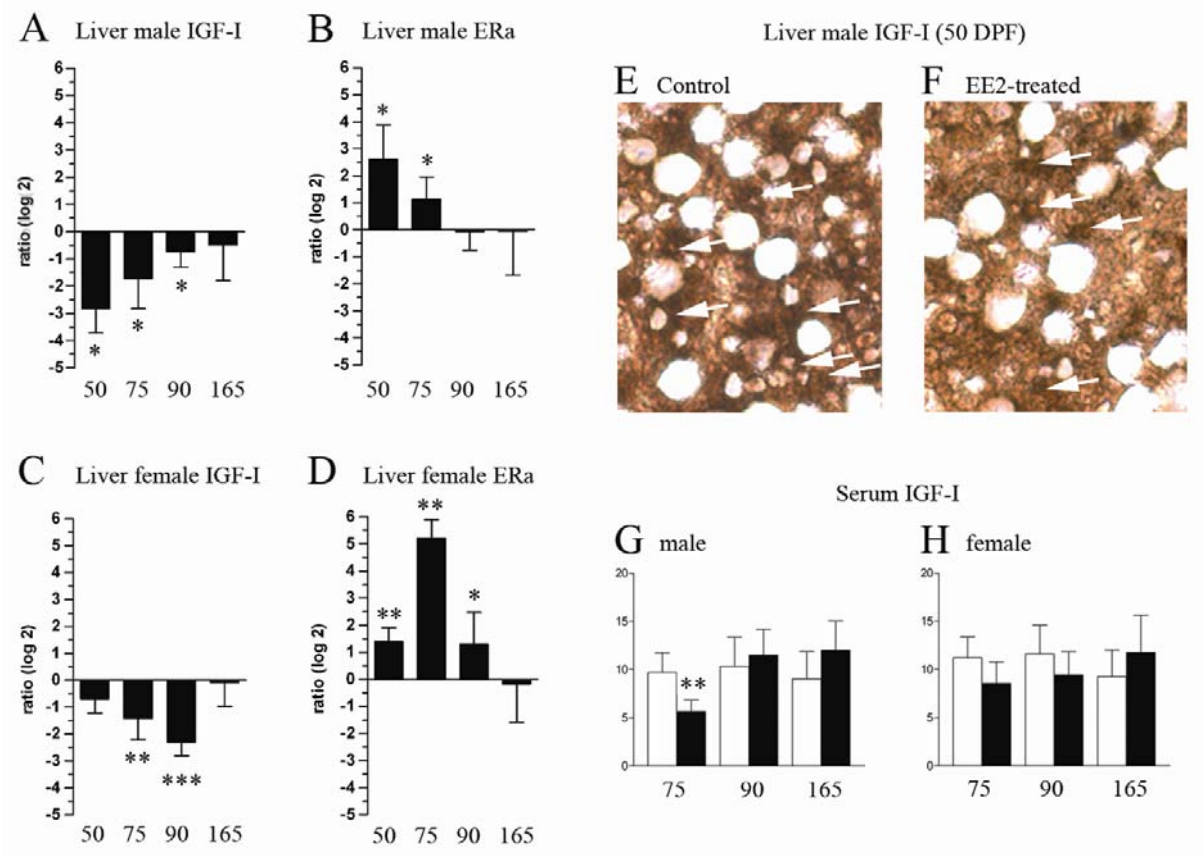

Fig. 9 Influence of EE2 exposure on IGF-I and ERα gene expression in liver and on IGF-I peptide levels in serum. (A-D) Relative changes (log 2) of IGF-I (A,C) and ERα (B,D) mRNA expression in EE2-treated tilapia as compared to age-matched control tilapia. (E,F) In situ hybridisation with IGF-I antisense probe of tilapia liver specimens in 50 DPF old control (E) and EE2-treated (F) male tilapia. White arrows point to IGF-I mRNA-expressing hepatocytes. (G,H) IGF-I peptide concentrations in serum determined by a fish-specific RIA in control (white columns) and EE2-treated (black columns) male (G) and female (H) fish. X-axis is labelled as DPF. Columns give mean values and bars SD. Significance level: * P=0.05, P=0.02, P=0.01; ** P=0.002, P=0.004; *** P=0.0003.

3.2.5. IGF-I mRNA and ERα mRNA levels in brain

In male brain, no significant change in the expression of IGF-I mRNA was detected throughout the experimental period (Fig. 10A). In female brain, however, at 75 DPF IGF-I mRNA was significantly (P= 0.001) reduced (3.45-fold). At 90 and 165 DPF, IGF-I mRNA was about the normal level (Fig. 10C).

At 75 DPF, in all regions of the female brain (Fig. 10F) the number of neurones showing IGF-I mRNA was largely reduced when compared to control (Fig. 10E). Brain ERα mRNA exhibited no significant changes at any experimental stage (Fig. 10B,D).

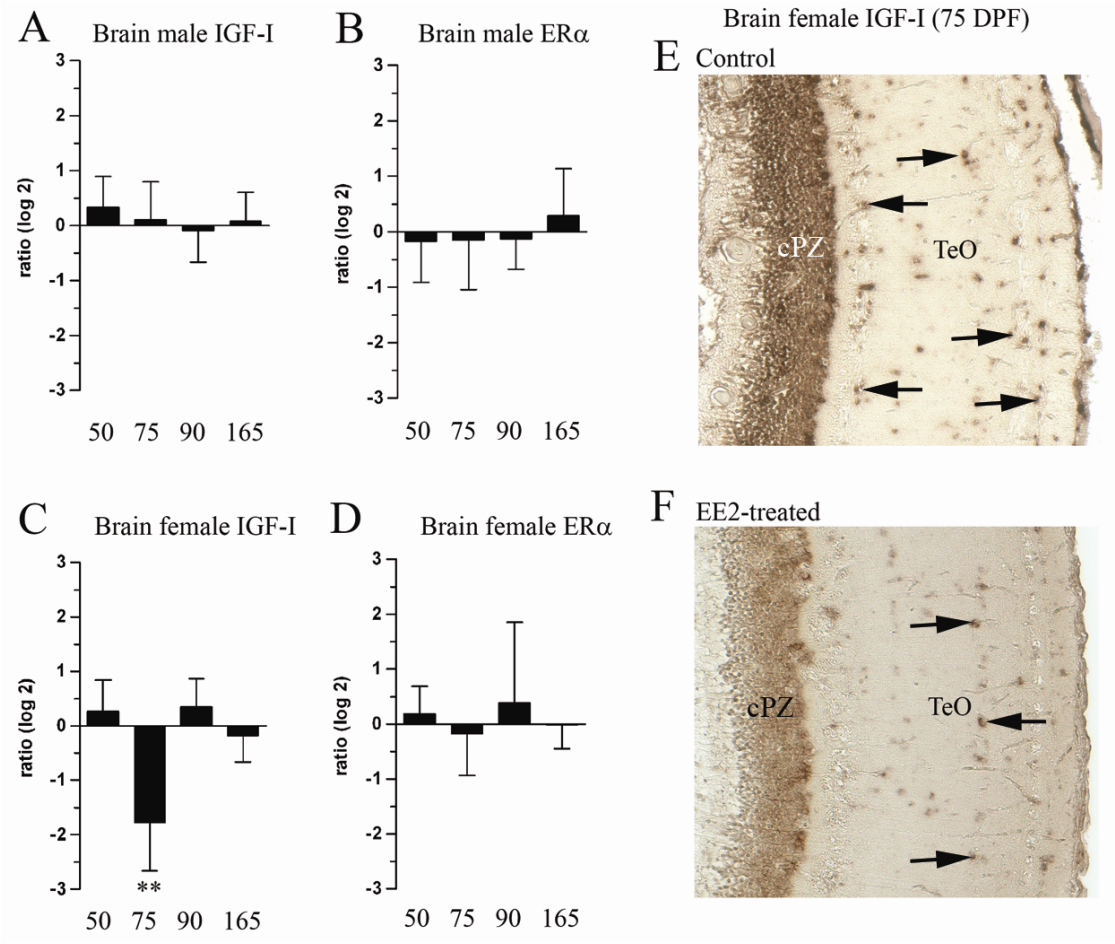

Fig. 10 Influence of EE2 exposure on IGF-I and ERα gene expression in brain. (A-D) Relative changes (log 2) of IGF-I (A,C) and ERα (B,D) mRNA expression in EE2-treated as compared to age-matched control tilapia. (E,F) In situ hybridisation with IGF-I antisense probe of tilapia brain specimens in 75 DPF old control (E) and EE2-treated (F) female tilapia. In the cell body layer of the periventricular zone (cPZ) and the tectum opticum (TeO) the number of IGF-I mRNA expressing neurons (black arrows) is largely reduced in the EE2-treated brain (F). X-axis is labelled as DPF. Columns give mean values and bars SD. Significance level: ** P= 0.001.

3.2.6. IGF-I mRNA and ERα mRNA levels in male and female gonads

No alteration in the expression of IGF-I was detected at 50 DPF (Fig. 11A,C). At 75 DPF, there was a significant (P=0.015) decrease (- 2.2-fold) in the IGF-I mRNA level in the female gonad and at 90 DPF a significant (P=0.0013) reduction (- 2.5-fold) of IGF-I mRNA in the male gonad. At the later stages, IGF-I mRNA reached the normal level. In situ hybridisation of male gonad at 90 DPF revealed that the number of IGF-I mRNA containing spermatogonia was markedly lower in EE2-treated (Fig. 11F) fish than in control (Fig. 11E). Less IGF-I mRNA signals were observed in granulosa cells of the ovaries in EE2-treated fish at 75 DPF (Fig. 11H). Furthermore, IGF-I mRNA in small oocytes as present in controls (Fig. 11G) was largely reduced. In the male gonads, a significant raise in ERα mRNA was found at 50 DPF (4.4-fold, P=0.015), 75 DPF (4.2-fold, P=0.028) and 90 DPF (2.6-fold, P=0.009) (Fig. 11B). In contrast, in the female gonad, a significant (P=0.015) increase in ERα mRNA was obtained only at 50 DPF by the 1.78–fold followed by a significant decrease (-1.79-fold, P=0.006) at 75 DPF. At 90 DPF, there was only a tendency (P=0.11) to decrease ERα mRNA. At 165 DPF, ERα mRNA was at the normal level.

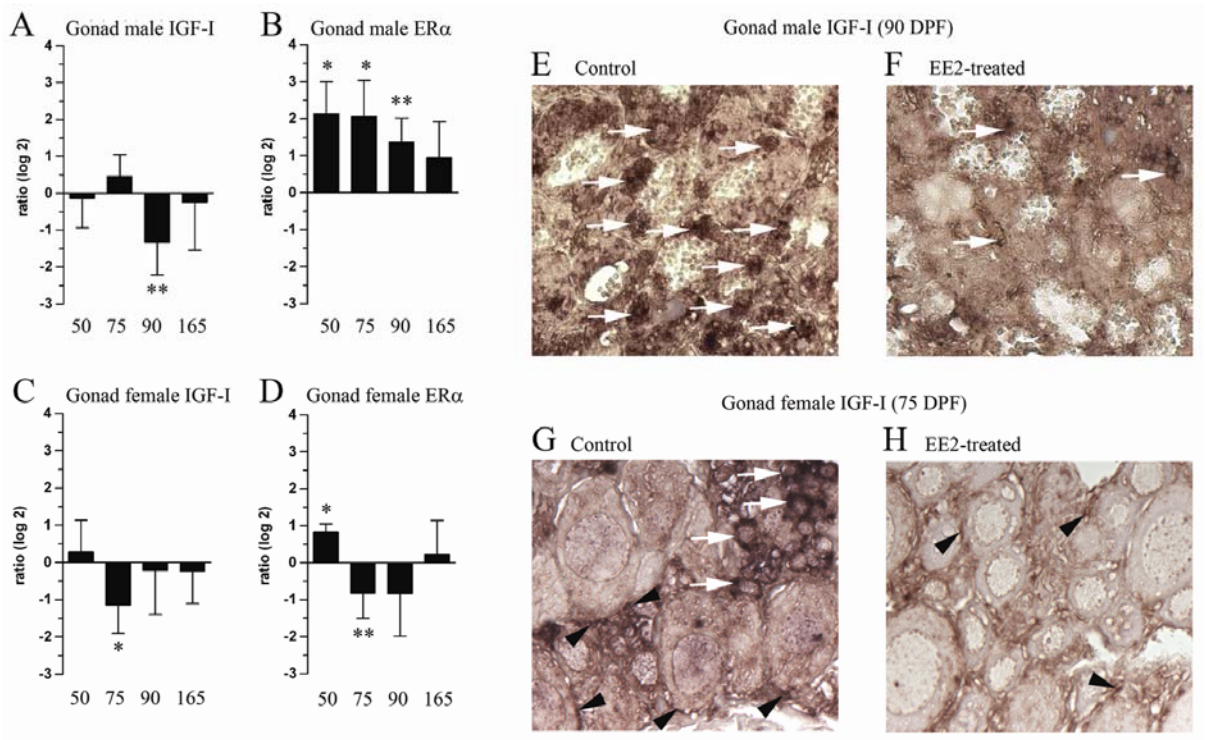

Fig. 11 Influence of EE2 exposure on IGF-I gene expression in male and female gonad. (A-D) Relative changes (log 2) of IGF-I (A,C) and ERα (B,D) mRNA expression in EE2-treated as compared to age-matched control tilapia. (E-H) In situ hybridisation with IGF-I antisense probe. (E,F) Male gonad at 90 DPF. Arrows point to IGF-I mRNA-expressing spermatogonia. (G,H) Female gonad at 75 DPF. Fewer IGF-I mRNA signals are found in granulosa cells (black arrow heads) and in small oocytes (white arrows) of EE2-treated fish (H) than in controls (G). X-axis is labelled as DPF. Columns give mean values and bars SD. Significance level: * P=0.028, P=0.015, ** P=0.006, P=0.009, P=0.0013.

3.2.7. IGF-I mRNA and ERα mRNA levels in gills

Significant changes in IGF-I mRNA expression were obtained only at 50 DPF in males where it was decreased by the 5.9-fold (P=0.02). No significant changes in IGF-I mRNA were detected in males lateron or in females at any experimental stage (Fig. 12A,C). Using in situ hybridisation, the number of IGF-I mRNA containing chloride cells in the gill filament epithelium was found to be reduced in EE2-treated males at 50 DPF (Fig. 12E,F). At 50 DPF, branchial ERα mRNA was significantly decreased in both sex, in males by the 27-fold (P=0.03) and in females by the 3.2-fold (P=0.03). At the later stages, no significant changes were obtained (Fig. 12B,D).

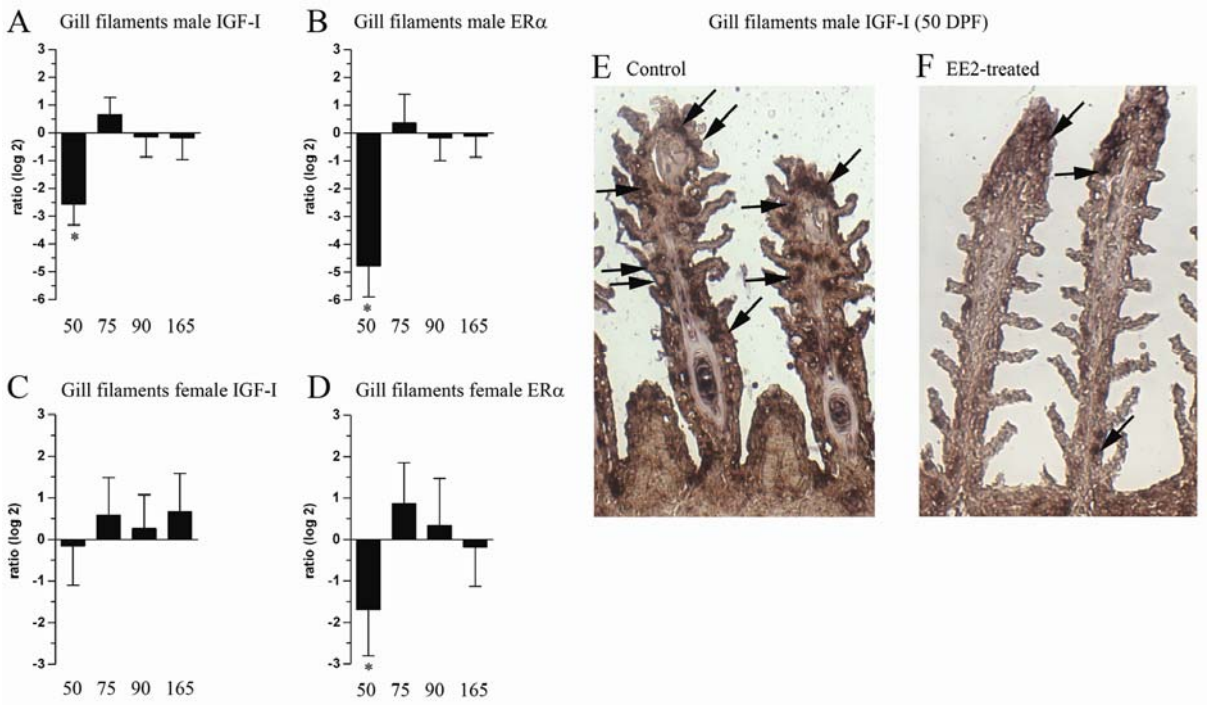

Fig. 12 Influence of EE2 exposure on IGF-I and ERα mRNA in gill filaments. (A-D) Relative changes (log 2) of IGF-I (A,C) and ERα (B,D) mRNA expression in EE2-treated as compared to age-matched control tilapia. (E, F) In situ hybridisation with IGF-I antisense probe in gill specimens of 50 DPF male tilapia. Black arrows point to IGF-I mRNA containing chloride cells in the gill filament epithelium. X-axis is labelled as DPF. Columns give mean values and bars SD. Significance level: * P=0.03, P=0.02.

3.2.8. Pituitary GH mRNA levels

Pituitaries could be dissected only at 75, 90 and 165 DPF. GH mRNA was significantly decreased after EE2 treatment in male pituitary at 165 DPF (P=0.0571) by the 2.33-fold (Fig. 13A) and in female pituitary at 75 DPF by the 2.27-fold (P=0.0571) and at 90 DPF to the 3-fold (P=0.0571) (Fig 13C). ERα mRNA was significantly (P=0.0061) raised to the 3.38-fold at 165 DPF in the male pituitary (Fig. 13B) and in the female pituitary to the 2.55-fold at 75 DPF (P=0.0159) and at 90 DPF (P=0.0012) to the 2.7-fold (Fig. 13D).

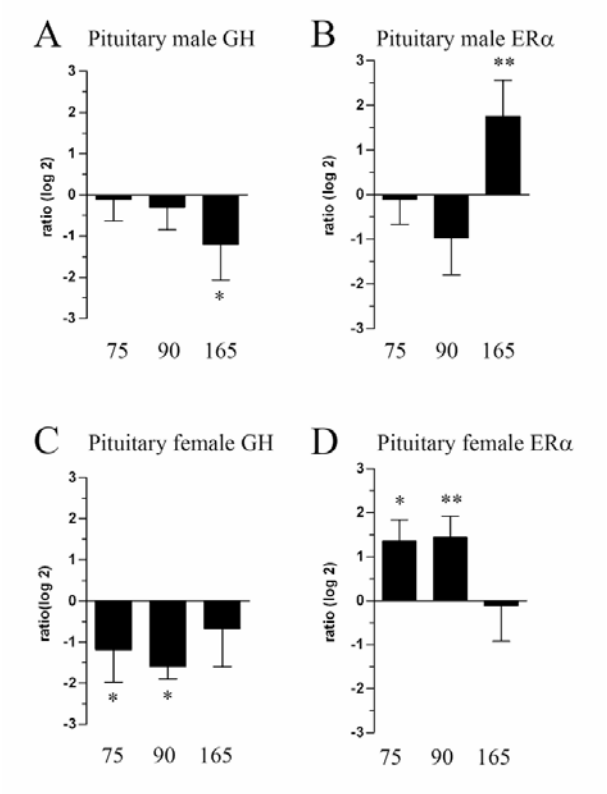

Fig. 13 Influence of EE2 exposure on GH and ERα mRNA levels in male (A,B) and female (C,D) tilapia pituitary revealed by real time PCR. Relative changes (log 2) of GH (A,C) and ERα (B,D) mRNA expression in EE2-treated as compared to age-matched control tilapia. X-axis is labelled as DPF. Columns give mean values and bars SD. Significance level: * P=0.0159, P=0.0571, ** P=0.0061, P=0.0012.

3.3. Environmental dose EE2 treatment

Our experiments with feeding of high doses of 17a-ethinylestradiol (EE2) indicated that developmental estrogen treatment impairs GH/IGF-I expression in fish, and that these effects persist. Consecutively, we studied the effects of exposure to low doses of EE2 on early developing tilapia.

3.3.1. Water concentration

Measurements of the actual EE2 concentrations after 36 h of exposure revealed in controls 0.088 ± 0.021 ng EE2/L, in the 5 ng EE2/L aquaria 4.34 ± 0.85 ng EE2/L, and in the 25 ng EE2/L aquaria 16.23 ± 5.74 ng EE2/L. Thus, the actual EE2 concentrations were below the applied ones.

3.3.2. Sex ratio

As obvious from Table 1, the female and male monosex fish populations after exposure to both 5 and 25 ng EE2/L exhibited a higher percentage of females at 75 and 100 DPF.

Female monosex	75 DPF	100 DPF	Male monosex	75 DPF	100 DPF
Control	78.7%	77.4%	Control	83.3%	81.7 %
5 ng EE2/L	83.3%	97.5%	5 ng EE2/L	78.4%	72.2%
25 ng EE2/L	91.6%	96.6%	25 ng EE2/L	75.0%	68.1%

Table 1 Sex ratio (%) of tilapia monosex populations after exposure to 5 and 25 ng EE2/L determined at 75 and 100 DPF.

3.3.3. Somatic indices

Exposure to EE2 affected growth more pronouncedly in male than in female fish. In male fish, from 50 DPF on both body length and weight were significantly reduced in fish exposed either in 5 or in 25 ng EE2/L. At the end of the experiment (100 DPF), in males body length (Fig.14A) was reduced by about 9.5% (5 ng EE2/L) and 11% (25 ng EE2/L) and body weight (Fig.14C) by about 25% at both EE2 concentrations. In female fish, both parameters showed only a trend to decrease. Here at 100 DPF, body length (Fig.14D) was reduced by about 3% (5 ng EE2/L) and 6.2% (25 ng EE2/L) and body weight (Fig. 14F) by about 14% at 25 ng EE2/L concentrations.

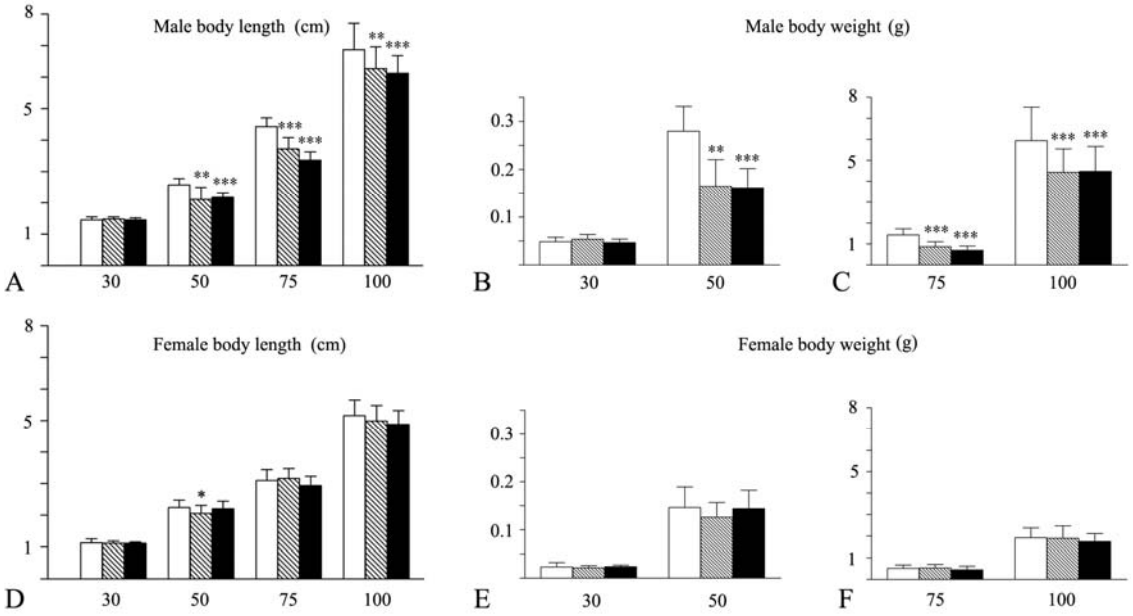

Fig. 14 Influence of EE2 exposure on fish growth throughout the experimental period. Body length (A,D, in cm) and weight (B,C,E,F, in g) in male (A,B,C) and female (D,E,F) tilapia.White columns: control, gray columns: 5 ng EE2/L, black columns: 25 ng EE2/L. X-axis is labelled as DPF. Columns give mean values and bars SD. Significance levels: * P <0.01; ** P<0.005; *** P<0.0005.

3.3.4. Impact on liver IGF-I and ERα gene expression

In both sexes, an initial (30 DPF) significant upregulation of IGF-I mRNA levels (Fig. 15A,C), even at the lower EE2 concentration (5 ng EE2/L), was observed (male: 2.82-fold, P=0.0007; female: 2.43-fold, P=0.003) which was accompanied in both sexes by elevated ERα levels (Fig. 15B,D) at 5 ng EE2/L (male: 1.77-fold, P=0.007; female: 2.24-fold, P=0.0007) and in females (Fig. 15D) also at 25 ng EE2/L (2.66-fold, P=0.0007). In males, already about 6 weeks of exposure (50 DPF), IGF-I gene expression (Fig. 15A) was highly significantly reduced at 25 ng EE2/L (-2.38-fold, P=0.0003) followed by a highly significant suppression of IGF-I mRNA levels at both concentrations, 5 ng EE2/L (-2.94-fold, P=0.0006) and 25 ng EE2/L (-4.17-fold, P=0.0006) which persisted until end of treatment (100 DPF). IGF-I gene expression dynamics were rather paralleled by significant down-regulations of ERα gene expression levels (Fig. 15B) at 75 DPF (5 ng EE2/L: -1.64-fold, P=0.0047; 25 ng EE2/L: -2.7-fold, P=0.0003) and 100 DPF (-2.08-fold, P=0.0082). Similar dynamics like in males were observed in females whereby the suppressed IGF-I mRNA levels (Fig. 15C) occurred later than in males: 75DPF (-1.41-fold, P=0.0093) and 100 DPF(-1.85-fold, P=0.015). Also in the female fish, the observed IGF-I mRNA levels coincided with a, however less pronounced, suppression (-1.59-fold, P=0.05) of ERα mRNA (Fig. 15D).

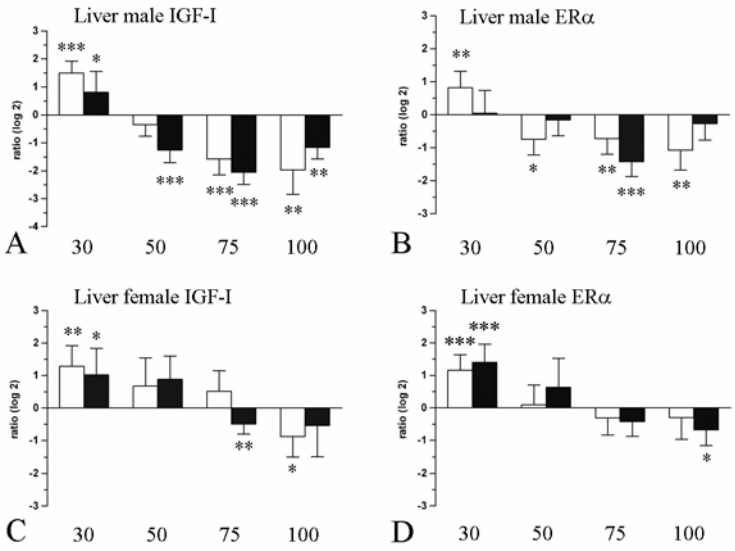

Fig. 15 Influence of EE2 exposure on IGF-I (A,C) and ERα (B,D) mRNA levels in tilapia male (A,B) and female (C,D) liver revealed by real time PCR. EE2-treated and age–matched control tilapia were used. X-axis is labelled as DPF. Columns give mean values and bars SD. White columns: 5 ng EE2/L, black columns: 25 ng EE2/L. Significance level: * P=0.015, P=0.02, P=0.05;** P=0.0012, P=0.003,P=0.0047, P=0.007, P=0.0082, P=0.0093; *** P=0.0003, P=0.0006, P=0.0007.

3.3.5. Impact on brain IGF-I and ERα gene expression

Exposure of male brain to EE2 significantly induced ERα gene expression (Fig. 16B) at the earliest time point investigated (30 DPF), at both concentrations (5 ng EE2/L: 1.76-fold, P=0.0499; 25 ng EE2/L: 1.73-fold, P=0.0379). Nevertheless, only at the high concentration of 25 ng EE2/L IGF-I mRNA levels (Fig. 16A) were significantly suppressed (-1.41-fold, P=0.0499), an effect that persisted until 50 DPF (-1.72-fold, P=0.0148) where ERα gene expression levels were still, but not significantly, elevated. Lateron, IGF-I gene was unaffected despite a significant down-regulation of ERα (-1.89-fold, P=0.0011) at the latest time-point investigated (100 DPF, Fig. 16B). In female brain, ERα gene levels (Fig. 16D) were suppressed over the whole treatment phase, but only rarely (5 ng EE2/L at 50 DPF: -1.53. P=0.0152; and 25 ng EE2/L at 100 DPF: -1.41-fold, P=0.0496) significantly. IGF-I gene expression levels (Fig. 16C) revealed an initial upregulation at 30 DPF (25 ng EE2/L: 2.16-fold, P=0.0012) followed by significant suppression at 50 DPF at both concentrations (5 ng EE2/L: -1.59-fold, P=0.0002; 25 ng EE2/L: -1.47-fold, P=0.0133). At 75 DPF, a recovery was observed at the higher concentration whereas at 5 ng EE2/L, a significant suppression (-2.38-fold, P=0.0001) was still observed followed by a significant elevation at 100 DPF (1.66-fold, P=0.0281).

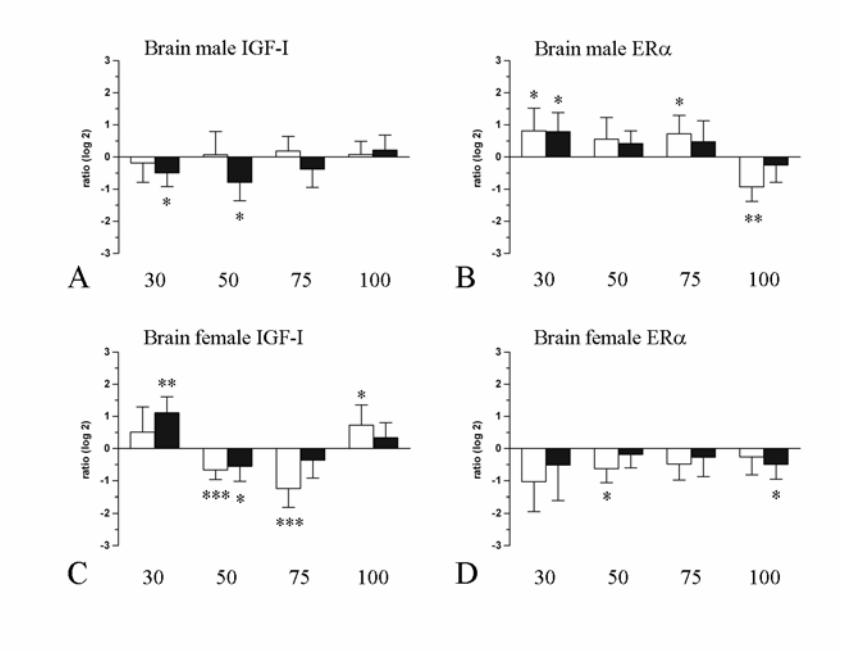

Fig. 16 In male brain, the ratios of IGF-I (A) and ERα mRNA (B) between EE2-treated and control tilapia changed over the experimental period. In female brain, IGF-I mRNA (C) is significantly reduced at 50 and 75 DPF and ERα mRNA (D) downregulated at 50 and 100 DPF. X-axis is labelled as DPF. Columns give mean values and bars SD. White columns: 5 ng EE2/L, black columns: 25 ng EE2/L. Significance level: * P=0.0133, P=0.0148, P=0.0152, P=0.0205, P=0.0281; P=0.0379, P=0.0496, P=0.0499; ** P=0.0011, P=0.0012; *** P=0.0001, P=0.0002.

3.3.6. Impact on gonad IGF-I and ERα gene expression

A marked sex-specific difference was observed in the gonads: in males (Fig. 17B), ERα was continuously, in part significantly, suppressed: initially, at 30 DPF at 25 ng EE2/L (-2.09-fold, P=0.0339), and later, at 50 DPF (-2.73-fold,P=0.0286) and 100 DPF (-1.95-fold, P=0.0037), at the low EE2 concentration (Fig. 17B). The continuously suppressed ERα gene expression levels were paralleled by continuously, in part significantly, down-regulated IGF-I gene expression levels (Fig. 17A) at 50 DPF (5 ng EE2/L: -3.04-fold, P=0.0286; 25 ng EE2/L: -6.07-fold, P=0.0286) and at 75 DPF (25 ng EE2/L: -2.33-fold, P=0.0303). Only at the latest time point investigated (100 DPF) when ERα was partly recovered (Fig. 17B) a significant upregulation of IGF-I mRNA (Fig. 17A) was observed at the higher EE2 concentration (25 ng EE2/L: 1.64-fold, P=0.0274). In females, no significant ERα gene expression change was detected (Fig. 17D). The IGF-I mRNA levels (Fig. 17C) were initially suppressed, but not significantly, and then showed a not significant but continuous elevation resulting in significantly elevated IGF-I mRNA levels at both EE2 water concentrations (5 ng EE2/L: 3.17-fold, P=0.0023; 25 ng EE2/L: 2.55-fold, P=0.0082).

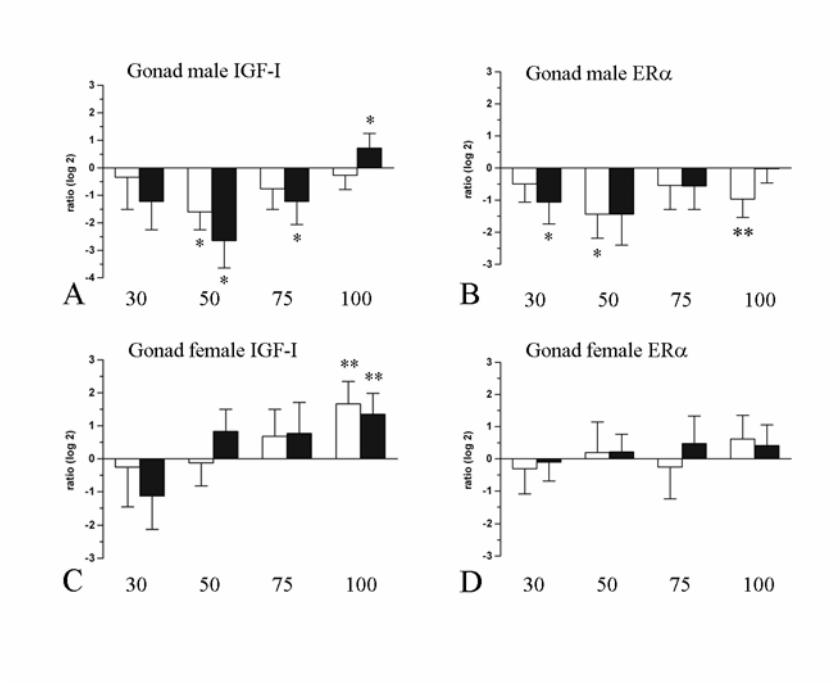

Fig. 17 At 50 and 75 DPF, IGF-I mRNA in male (A) gonad of EE2-treated tilapia as revealed by real time PCR remains downregulated. At 100 DPF, in female (C) and in male (A) gonad the IGF-I mRNA level is significantly increased. ERα mRNA is significantly suppressed in the male gonads (B) at 30, 50 and 100 DPF. In the female gonad, ERα mRNA (D) remained unchanged through the whole experiment. X-axis is labelled as DPF. Columns give mean values and bars SD. White columns: 5 ng EE2/L, black columns: 25 ng EE2/L. Significance level: * P=0.0274, P=0.0286 , P=0.0303, P=0.0339; ** P=0.0023, P=0.0037 , P=0.0082.

3.3.7. Impact on gills IGF-I and ERα gene expression

In male gills, alteration of the ERα gene levels (Fig. 18B) were only observed at the lower EE2 concentration (5 ng EE2/L) and these were significant upregulations at 30 DPF (1.73-fold, P=0.0281) and 75 DPF (2.09-fold, P=0.0229). No IGF-I mRNA gene expression alteration (Fig. 18A) was observed at the lower EE2 concentration. But in contrast, at 25 ng EE2/L, a continuous suppression of IGF-I mRNA was detected starting at 30 DPF, at 50 DPF it was significant (-1.93-fold, P=0.0019), followed by a not significant upregulation at 100 DPF (Fig. 18A). In female gills, ERα mRNA (Fig. 18D) was lowered, whereby the suppression at the higher EE2 concentration occurred earlier at 50 DPF (-2.33-fold, P=0.004) than at the lower concentration, started at 75 DPF (5 ng EE2/L: -1.75-fold, P=0.004; 25 ng EE2/L: -2.22-fold, P=0.0002) and persisted, but not significantly, until end of treatment. Whereas no alteration of IGF-I mRNA levels (Fig. 18C) occurred at the low EE2 concentration, a significant down regulation was observed at 25 ng EE2/L at 50 DPF (-2.33-fold, P=0.001) followed by a significant elevation at 75 DPF (1.72-fold, P=0.0111) and a recovery at 100 DPF.

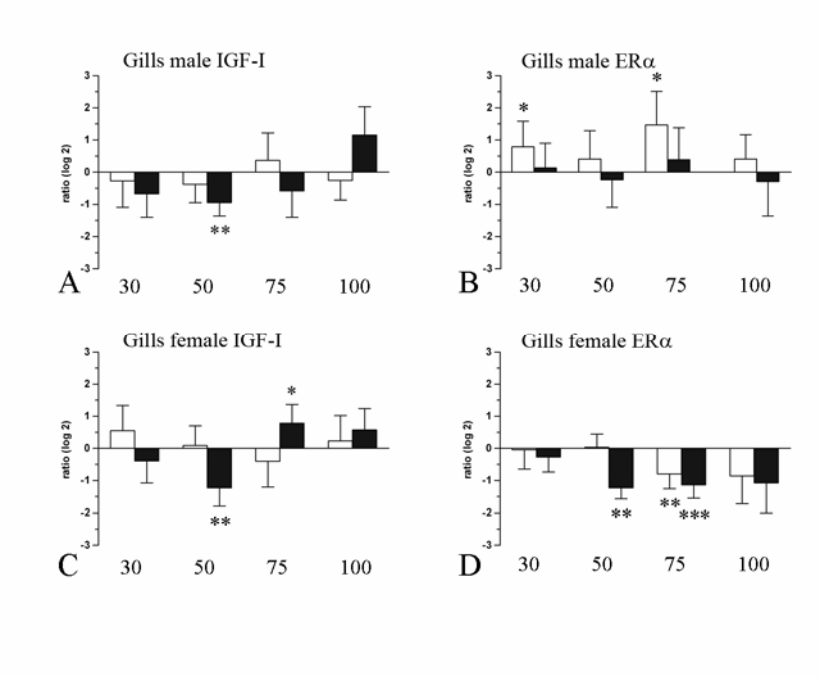

Fig. 18 Impact of EE2-treatment on IGF-I mRNA (A,C) and ERα mRNA (B,D) in gills. A significant decline in IGF-I mRNA expression was found only at 50 DPF in males (A) but no significant changes in IGF-I mRNA were detected in males lateron. In females at 50 DPF IGF-I mRNA (C) was downregulated with lateron compensatory recovery. In females, branchial ERα mRNA (D) was significantly decreased at 50 and 75 DPF. But in males, significant upregulations at 30 and 75 DPF were observed (B). X-axis is labelled as DPF. Columns give mean values and bars SD. White columns: 5 ng EE2/L, black columns: 25 ng EE2/L. Significance level: * P=0.011, P=0.0229, P=0.0281; ** P=0.001, P=0.0019, P=0.004, P=0.002; *** P=0.0002.

4. Discussion

Nothing is known about the potential interference of estrogen(s) and IGF-I during fish development. In our first experimental approach, we have used EE2-feeding from 10 to 40 DPF, a period that includes the sensitive period of gonad differentiation. The effects of this treatment have been determined at 50, 75, 90 (puberty) and 165 (adult) DPF and will be first discussed.

EE2 feeding led to a lasting decline in growth that was most pronounced at the end of the experiment (165 DPF) i. e. about three months after end of the treatment, in the young adult individuals. Then, body weight was reduced compared to controls in males by about 46% and body length by 19.5%, and in females by about 40% and 15%, respectively. Thus, EE2-feeding for about one month during the sensitive phase of sexual development resulted in severe and persistent growth impairment in both sex.

Although growth reducing effects of continuous estrogen exposure on developing fish have been shown in some studies, less information is available whether estrogen exposure during specific developmental stages leads to altered growth later in life. Treatment of embryonic trout with estrogen-receptor-binding alkylphenols until 21 DPF resulted in a permanently suppressed growth until 400 DPF (Ashfield et al., 1998). This finding agrees with our observation that developmental estrogen exposure evoked a persistent reduction of tilapia growth. The question is if this permanent growth-suppressing effect is caused by an interaction with the GH/IGF-I system, be it directly and/or indirectly. To this end, we analysed parameters of the GH/IGF-I system, in a series of target and effector organs, such as liver, gonads, brain, pituitary and gills, and both at the transcriptional and translational level.

Feeding of tilapia with EE2 during early development resulted in a significant decrease in circulating serum IGF-I by about 30% at 75 DPF, i.e. about one month after end of treatment. This decline was highly significant in males, and in females showed a clear tendency. In agreement, the somatic indices were more pronouncedly lowered in males than in females. The decreased serum IGF-I was in parallel with a high and significant decrease in IGF-I mRNA in liver of both sexes, the main source of endocrine IGF-I, and a reduced number of IGF-I mRNA expressing hepatocytes as shown by in situ hybridisation. Further, the decline in hepatic IGF-I synthesis was accompanied by a significant induction of ERα mRNA which was most pronounced at the time of the strongest decline of hepatic IGF-I expression. ERα mRNA was induced by EE2 treatment also in other tissues such as pituitary

or brain, and this response represents the well-characterised autoregulatory effect of estrogens on their own receptors, as it has been shown also for other fish species, e.g., (Filby et al., 2007). However, it needs to be emphasised that the association between the upregulation of ERα and the altered expression of IGF-I, as observed in our study, does not implicate a mechanistic link between the two observations. However, even without assuming a causative role of the EE2-induced activation and upregulation of the ER pathway, our results suggest that administration of EE2 during early development exerts a long-term suppressive effect on hepatic IGF-I expression and synthesis. Previous *in vivo* and *in vitro* studies in adults of different fish species also reported an estrogen-associated decrease in hepatic IGF-I mRNA (Riley et al., 2004; Carnevali et al., 2005; Filby et al., 2006) or in serum IGF-I (Arsenault et al., 2004; McCormick et al., 2005).

However, effects obtained in the other studies occurred during ongoing estrogen treatment while the effects observed in our study represent lasting effects of exposure earlier in life. The EE2 effect on IGF-I of tilapia was partly gender-specific. For instance, the EE2-induced decrease in liver IGF-I mRNA appeared earlier (50 DPF) in males than in females (75 DPF). Interestingly, this was paralled by a later increase in ERα mRNA expression in females, suggesting a causative link between these events, although we cannot prove this on the basis of our data. Sex-specific responses of the IGF-I system of fish to estrogens have been reported also by Filby et al. (2006), who found that the hepatic expression of IGF-I in adult fathead minnow exhibited a high and significant decrease in males exposed to E2 but only an insignificant one in females. Gender differences in the response of IGF-I to estrogens may indicate that the estrogen effects on IGF-I expression in organs such as the liver do not not only result from a direct, local crosstalk between the two hormone systems, but interactions at the hypothalamus-pituitary level and subsequent systemic changes may be involved as well. Interestingly, brain IGF-I mRNA was responsive to developmental EE2 treatment only in female tilapia. Here the suppression of IGF-I expression obtained by real-time PCR was paralled by an overall decrease in IGF-I mRNA signals in neurons as found by in situ hybridisation.

Furthermore, fish of either sex fed with EE2 exhibited a significant decrease in GH mRNA which was accompanied by a significant upregulation of the ERα. Evidence for effects of estrogens on the fish pituitary GH gene is conflicting. While in some studies no effects of E2 on GH mRNA were revealed (Melamed et al., 1998; Filby et al., 2006), others report that E2 stimulated GH synthesis and secretion, but not gene transcription (Holloway and Leatherland, 1997; Zou et al., 1997). The discrepancy between these studies and the present

one may be due to the different estrogens used, to different modes of application or to hormone application at different life stages or physiological states of the fish. The reduction in pituitary GH mRNA as observed here may well be caused by a direct effect of EE2 on the pituitary GH cells. However, EE2 may have also suppressed GH release at the hypothalamic level because E2 increased the expression of somatostatin-14 in goldfish brain (Canosa et al., 2002).

In the gonads, IGF-I occurs during the juvenile and adult stage in testes in spermatogonia, spermatocytes, Sertoli and Leydig cells (Le Gac et al., 1996; Reinecke et al., 1997; Berishvili et al., 2006b) and in the ovary in small and previtellogenic oocytes and in follicular granulosa and theca cells (Schmid et al., 1999; Perrot et al., 2000; Berishvili et al., 2006b). In Japanese eel cultured testes, IGF-I stimulated spermatogenesis induced by 11-ketosterone (Nader et al., 1999). In rainbow trout, testicular IGF-I increased after GH-treatment (Le Gac et al., 1996; Biga et al., 2004) and both GH and IGF-I stimulated the incorporation of thymidine into spermatogonia and primary spermatocytes (Loir, 1999). In the ovary of different fish species, IGF-I stimulated thymidine incorporation in vitellogenic follicles (Srivastava and Van der Kraak, 1994), promoted oocyte maturation (Kagawa et al., 1994; Negatu et al., 1998) and selectively influenced the production of sex steroids in theca and granulosa cells (Maestro et al., 1997). Hence, IGF-I most likely acts as paracrine/autocrine regulator of fish spermatogenesis and oocyte proliferation and maturation in interaction with steroid hormones.

Feeding with EE2 caused a significant reduction in IGF-I mRNA in both ovaries and testes. The downregulation occurred earlier in female than in male gonad. In agreement with the PCR results, in situ hybridisation showed in testes a decrease in the number of IGF-I mRNA containing spermatogonia, and in ovary a reduction of IGF-I expression in small oocytes and granulosa cells after EE2 treatment.

Remarkably, the ERα mRNA showed a long-term induction by EE2 feeding only in the testes while in the female gonad a significant downregulation of the ERα mRNA was obtained at 75 DPF after an initial upregulation at 50 DPF. Similarly, in adult fathead minnow exposed to E2 for 14 days, Erα mRNA was also increased in male and decreased in female gonad (Filby et al., 2006). Thus, ER signaling pathway and IGF-I expression of male and female gonads show differential responses to EE2 exposure what might indicate that in this case the EE2 effects on IGF-I are not mediated through the ER pathway.

Overall, from the present results, two options for explaining the impairing effects of estrogens on growth, differentiation and function of fish gonads are likely: They may be

exerted via suppression of IGF-I production in liver resulting in a lowered level of circulating (endocrine) IGF-I and/or by the reduction of autocrine/paracrine IGF-I expression within the gonads as discussed above.

In gill filaments, only males exhibited a significant decrease in IGF-I mRNA at 50 DPF that is reflected at the cellular level by a reduction of the number of IGF-I mRNA containing chloride cells, while no significant changes were present throughout the experimental period in females. The results at 50 DPF, i.e. 10 days after end of the treatment, agree in part with those obtained in the adult cyprinid fathead minnow exposed to E2 for 14 days (Filby et al., 2006): IGF-I mRNA was amplified only in the gills of some fish and, here a down-regulation was found in both sexes. IGF-I seems to have a physiological impact on smoltification. The chloride cells of the filament epithelium not only express Na^+,K^+-ATPase (McCormick, 1996) but in developing and adult fish also IGF-I mRNA (Reinecke et al., 1997; Radaelli et al., 2003; Berishvili et al, 2006a). In tilapia, the importance of local IGF-I expression is stressed by its very early appearance in chloride cells around 6-7 DPF (Berishvili et al. 2006a). In several fish species, evidence has been presented that both circulating (liver-derived) IGF-I (Madsen and Bern, 1993; Shepherd et al., 1997; Inoue et al., 2003) and autocrine/paracrine IGF-I from the chloride cells (Sakamoto and Hirano, 1993; Biga et al., 2004) mediate the osmoregulatory actions of GH. Previous studies have reported that E2 impairs osmoregulation in salmonid (Arsenault et al., 2004; Madsen et al., 2004; McCormick et al., 2005) and non-salmonid (Vijayan et al., 2001) fish, and suppressed plasma levels of IGF-I were thought to be the underlying mechanism (McCormick et al., 2005). The present results not only support this idea but also suggest a direct effect of estrogens on IGF-I production in gill filaments in addition to the endocrine route. This is not only indicated by the present PCR results but also by the observed decrease of IGF-I mRNA in chloride cells as revealed by in situ hybridisation.

The findings from our EE2 feeding experiments on developing fish are in line with some earlier findings on adult fish that estrogen(s) are able to modulate IGF-I transcription and translation in liver (Riley et al., 2004; Carnevali et al., 2005; Filby et al., 2006) or decrease serum IGF-I (Arsenault et al., 2004; McCormick et al., 2005).

In addition, the study provides evidence that estrogens applied during early development

a) change the IGF-I system in liver and, concomitantly, circulating IGF-I and, thus, influence the endocrine route of IGF-I action,

b) impair local IGF-I in other organs by changing IGF-I expression within the organ-specific cells as shown by in situ hybridisation, and

c) that the IGF-I response is associated with a change of pituitary GH expression.

Thus, the estrogen effect on IGF-I seems to involve both direct (autocrine/paracrine) interactions in peripheral organs as well as indirect (endocrine) effects via modulation of the brain-pituitary GH system. Our results also for the first time provide evidence that developmental estrogen exposure can have long-lasting effects on the GH/IGF-I system.

As first and successful approach we used a dietary EE2 concentration in the range used for intended feminisation of tilapia in aquaculture (Piferrer 2001) to reveal whether interactions between the estrogen and the GH/IGF-I system in principal can take place. Dietary application of estrogens, similar to injection of estrogens that has also often been described, therefore represents an articifical exposure situation which is useful to provide principal information on mechanisms and targets but not to assess the environmental hazard of estrogens. Thus, in a second set of experiments we have investigated whether environmentally relevant concentrations of EE2, i.e. 5 and 25 ng EE2/L surrounding water, also affect the fish IGF-I system.

To this end, we have incubated both a male and a female monosex populations from 10 to 100 DPF at the concentrations 5 ng/L EE2 and 25 ng/L EE2 and determined the effects of EE2 exposure at 30, 50, 75 and 100 DPF.

In our study we have exposed developing tilapia with low concentrations of EE2 in the surrounding water. In numerous countries, remarkable concentrations of EE2 have been determined in the environment. In Germany, concentrations of EE2 in rivers ranged between 1 ng/L (Hintemann et al., 2006) and 5 ng/L (Kuch and Ballschmiter 2001) while in WWTP effluents they were 3 ng/L (Hintemann et al., 2006), 9 ng/L (Kuch and Ballschmiter, 2001) and 15 ng/L (Ternes et al., 1999). Similarly, EE2 concentrations in effluents in the UK were around 7 ng/L (Desbrow et al., 1998), in the Acushnet River Estuary in the USA 4.7 ng/L (Zuo et al., 2006), in a river in Taipei up to 15 ng/l (Chen et al., 2007). In France and the Netherlands 3-4 ng EE2/L were detected in coastal water and rivers and 4-8 ng EE2/L in effluents (Belfroid et al., 1999; Cargouet et al., 2004). In a Canadian study even up to 42 ng EE2/L were reported in effluents (Ternes et al., 1999). Thus, the actual exposure concentrations of EE2, i.e. on average

4.34 ng EE2/L and 16.23 ng EE2/L, used in our study, are well within the range determined for EE2 in the environment.

Sex ratio had shifted at 75 and 100 DPF. Both the female and male monosex fish populations exhibited a higher percentage of females after exposure to both 5 and 25 ng EE2/L. Thus, as the high EE2 doses applied in our feeding experiment, environmentally relevant concentrations of EE2 also led to feminisation while no hermaphrodites were found. In further parallel to the effects of EE2 feeding growth and length of the exposed fish were also decreased. The results of our exposure experiments support those obtained in zebra fish that was also exposed to environmentally relevant concentrations of EE2 (up to 10 ng EE2/L) and also showed impaired juvenile growth (Schafers et al., 2007).

In both sex, after an initial significant upregulation of the IGF-I mRNA levels at both EE2 concentrations (5 and 25 ng EE2/L) the expression of IGF-I mRNA in liver was reduced. This is in agreement with previous studies where adults of different fish species were exposed to E2 resulting also in an estrogen-associated decrease in hepatic IGF-I mRNA (Riley et al., 2004; Carnevali et al., 2005; Filby et al., 2006). The suppression of liver IGF-I mRNA in both sexes was accompanied by a downregulation of the ERα. The latter result is in contrast to those obtained by our EE2 feeding experiments and those described in adult fathead minnow (Filby et al., 2006) where the decrease in IGF-I mRNA was accompanied by an upregulatuion of the ERα. It is conceivable that the long-term exposure to EE2 in our experiments - that first led to an upregulation of the ERα as expected - may have caused the downregulation possibly indicating some over-compensation.

Under exposure to low concentrations of EE2, the expressions of the genes investigated showed some sex-specificity in the gonads. In males, ERα was continuously downregulated while in females no significant alterations occurred. IGF-I mRNA in testes was transiently decreased but in females after an initial trend to decrease IGF-I mRNA was upregulated at 100 DPF. Recent evidence obtained in zebra fish exposed to low concentrations of EE2 (up to 10 ng EE2/L) in surrounding water indicated not only a reduction of juvenile growth but also an impairment of sexual maturity, adult fecundity and fertilisation success (Schafers et al., 2007). Based on the present results it is assumed that the observed impairing effects of EE2 on reproductive processes may be due to a downregulated expression of IGF-I mRNA in the gonads, particularly in testes. Furthermore, it is likely that the feminisation found in the present study is a further parameter that impairs fertilisation success.

Individual growth has consequences for demographic parameters such as age-specific survival, time to maturation or fecundity. Thus, our findings obtained by both experimental approaches point to a potentially important mechanism through which environmental estrogens, in addition to their direct effect on fish reproduction, could alter population growth of fish species.

Future research has to address the mechanism(s) through which estrogen(s) act to suppress pituitary GH and local IGF-I expression in liver and extrahepatic sites. In particular, given our findings on the correlation between ER and IGF-I changes, it would be important to examine whether the EE2 effects on the GH/IGF-I system are mediated through the ER pathway and/or through other mechanisms.

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Publications

Shved N, Berishvili, G, D'Cotta H, Baroiller J-F, Segner H, Eppler E, Reinecke M.
Ethinylestradiol (EE2) differentially interferes with insulin-like growth factor I (IGF-I) in liver and extrahepatic sites of male and female bony fish.
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A survey on the expression of IGF-I in the early developing bony fish with special emphasis on the tilapia, *Oreochromis niloticus*.
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